

**Università degli studi di Napoli “Federico II”**

**Dipartimento di agraria**

**Dottorato di ricerca in Scienze e Tecnologie delle Produzioni Agro-Alimentari**

**XXV ciclo**



**PROTEOMIC AND IMMUNOCHEMICAL CHARACTERIZATION  
OF FOOD ALLERGENS OF PLANT ORIGIN:  
THE HAZELNUT (*Corylus avellana*) CASE OF STUDY**

**Supervisor: Prof. Pasquale Ferranti**

**Co-supervisor: Dott. Gianfranco Mamone**

**Coordinator: Giancarlo Barbieri**

**PhD student: Chiara NITRIDE**

**2012/2013**

*"Everything should be as simple as possible, but not simpler."  
Einstein*

## ABSTRACT

The application of proteomics strategies to identify and characterize food allergens has been defined ‘allergenomics’ in 2005 by the Division of Medical Devices, National Institute of Health Science in Japan. The main purposes are to achieve more detailed and comprehensive characterization of food allergens, to define markers for “hidden allergens” monitoring in complex food matrices and to develop more specific and standardized protocols for clinical diagnosis and immunotherapy.

Hazelnut (*Corylus avellana*) is one of the most common causes of lifetime lasting IgE-mediated food allergy. Immune reactions to hazelnut range from mild oral allergy syndromes to severe life-threatening anaphylaxis. Hazelnut allergy is more frequent in infancy than in adulthood and its prevalence varies among countries. In the years different studies aimed to identify the hazelnut allergic determinant(s) have been carried out. Several important hazelnut seeds storage proteins have been identified and characterized as allergens such as Cor a 1.04, major allergen in the Northern Europe related to 65 patients sensitized to birch (Bet v 1, *Betula verrucosa*); 11S globulin (Cor a 9, with 30–40 kDa acidic and 20–25 kDa basic chain bounds together *via* disulfide bridge), described as the major non-pollen related allergen in United States; 48 kDa-glycoprotein (Cor a 11); *ns*-LTP (Cor a 8), supposed to be the major allergen in Southern Europe, and 2S albumin (Cor a 14). The predominance of the specific allergens appears to be associated to the geographical origin of allergic subjects. The complexity of the hazelnut allergen pattern challenges a clear definition of both the allergens and the allergenic determinants (epitopes).

Mass spectrometry-based strategies, combining two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and liquid chromatography (LC) techniques for proteins resolving, were developed with the aim of identifying and characterizing proteins that can function as markers for hazelnut allergens. These achievements made possible the identification of still not described hazelnut proteins has been possible by homology comparison with gene products arising from other seeds, even with a relatively low sequence coverage; the complete characterization of the both oligosaccharide chains of the 48 kDa glycoprotein, Cor a 11 allergen; the improvement of the strategy for *ns*-lipid transfer protein, Cor a 8 allergen, purification.

Immunochemical assays were exploited to investigate the pattern of the immunological response of hazelnut allergic children from Campania region. Fifteen out fifteen children’s sera were immunoreactive to a protein that has not been annotated in database so far. The mass spectrometry-based characterization, also including the “*de novo*” sequencing of tryptic peptides, provided evidence of the high homology degree between the unknown IgE-binding polypeptide and the 11S globulin-like storage proteins that are expressed in several other seeds. In spite of a low sequence similarity, the new allergen, share structural traits with the hazelnut 11S globulin-like proteins (Cor a 9).

The simulation of *in vitro* gastrointestinal digestion of the pure isoform of 11S globulin-like and the dot-blot analysis with sera of allergic patients showed that the total peptide digest of crude proteins and pure 55 kDa IgE binding proteins retained IgE-reactivity. These finding suggest the presence of linear IgE epitope(s). Further proteomic investigations are required to identify IgE-binding domains of putative hazelnut allergen(s) resistant to digestion.

# TABLE OF CONTENTS

<b>1. State of the art</b>	<b>6</b>
1.1 Food Allergies	7
1.2 Allergy to Tree Nuts and Edible Seeds: Hazelnut allergy	9
1.3 “Allergenomics”	11
1.3.1 Identification of allergens in transformed products	14
1.3.2 Effect of gastrointestinal proteolysis on allergens	16
1.4 References	18
<b>2. Section 1. Proteomic characterization of hazelnut allergens</b>	<b>24</b>
2.1 Aim of the study	25
2.2 Optimization of proteomic sample preparation procedures for comprehensive protein characterization of hazelnuts system	25
2.2.1 Materials	25
2.2.2 Methods	25
2.2.2.1 Protein Extraction	25
2.2.2.2 SDS-PAGE analysis	25
2.2.2.3 Two-dimensional IEF/SDS-PAGE	26
2.2.2.4 Protein in-gel digestion	26
2.2.2.5 Gel filtration isolation of low molecular weight proteins	26
2.2.2.6 RP-HPLC separation of hazelnut proteins	26
2.2.2.7 MALDI-TOF MS	26
2.2.2.8 Shotgun proteomic analysis by nano-HPLC-ESI MS/M	26
2.2.3 Results and discussion	27
2.2.3.1 Electrophoretic Comparison of Seed Proteins Among Varieties by 1D and 2D-PAGE	27
2.2.3.2 Isolation and characterization of Low-Molecular Weight Proteins	27
2.2.3.3 RP-HPLC fractionation of PBS extracted proteins from raw hazelnut	29
2.2.3.4 Shotgun proteomic analysis	30
2.3 Characterization of N-glycans of the 48 kDa glycoprotein, Cor a 11 by hydrophilic interaction liquid chromatography and MALDI-TOF MS	30
2.3.1 Material	30
2.3.2 Methods	30
2.3.2.1 HILIC glycopeptide enrichment	30
2.3.2.2 Deglycosylation	30
2.3.2.3 MALDI-TOF analysis	31
2.3.3 Results and discussion	31
2.4 Purification and characterization of <i>non-specific</i> Lipid Transfer Protein ( <i>ns-LTP</i> ), Cor a 8. (IFR- Institute of Food Research).	32
2.4.1 Materials	32
2.4.2 Methods	32
2.4.2.1 Preparation of protein extract for immunoblotting analysis	32
2.4.2.2 SDS-PAGE analysis	32
2.4.2.3 Immunoblotting analysis with anti rCor a 8	32
2.4.2.4 Purification of ns-LTP	32
2.4.3 Results and discussion	33
2.5 Conclusion	35
2.6 References	36
<b>3. Section 2. Proteomic characterization of the alkaline subunit of a hazelnut (<i>Corylus avellana</i>) 11S globulin isoform, a new putative food allergen</b>	<b>37</b>
3.1 Aim of the study	38
3.2 Materials	38
3.2.1 Patients	38
3.3 Methods	38
3.3.1 Immunoblot for IgE-binding assay and immunoblot inhibition	38
3.3.2 Purification of the IgE-binding protein	39
3.3.3 MS/MS Analysis and de novo Sequencing	39

3.4	Results and discussion	39
3.5	Conclusion	46
3.6	References	48
4.	Section 3. Simulated gastrointestinal digestion of hazelnut proteins	49
4.1	Aim of the study	50
4.2	Material	50
4.3	Methods	50
4.3.1	Preparation of human BBM	50
4.3.2	Gastric–pancreatic and BBM digestion	50
4.3.3	Dot-blot analysis	50
4.3.4	4.3.4 RP-HPLC analysis	50
4.4	Results and discussion	50
4.5	Conclusion	52
4.6	References	53
Appendix I	Short-Term-Mobility - Infogest COST Action	54
I.1	Introduction	55
I.2	Material	55
I.3	Method	55
I.3.1	In vitro digestion model	56
I.3.2	SDS-PAGE analysis	56
I.4	Results	56
I.5	References	57
Appendix II.	Scientific production	58

**1.**  
**State of the art**

## 1.1 Food Allergies

Food allergy has become an important health problem especially in industrialised countries. It is estimated that IgE mediated food allergy affects about 3-4% of the adult population and up to 8% of the children in developed western countries (Monaci et al., 2006; Schnell and Herman, 2009). Although any food may elicit allergic reaction, relatively few foods are responsible for the majority of food allergic reactions: cow's milk, egg, peanut, tree nuts (for example, walnuts, pecans, almonds, and cashews), fish, shellfish, soy, and wheat. These are often referred to as "the big eight."

The term "food allergy" has no universal definition. Many consumers consider "food allergies" all of the *adverse reactions to food* consisting in an abnormal response of the immune system that occurs after the ingestion, contact or inhalation of a food (Fernández-Rivas and Miles 2004). In 1984 the American Academy of Allergy and Immunology with the National Institute of Allergy and Infectious Disease classified the *adverse food reactions* into: food allergy (hypersensitivity) and food intolerance depending on the existence or not of an underlying immunological mechanism, respectively (Anderson and Song 1984). In 1995 the European Academy of Allergy and Clinical Immunology (EAACI) introduced a different classification of adverse reaction to food: toxic and non-toxic reactions. Toxic immunological reactions occur in consequence to the exposition to high dose of anti-nutritional factors and are not related to the individual susceptibility. Differently the non-toxic reactions are classified into: *food allergy (hypersensitivity)* which can occurs when a susceptible individual produces IgE antibodies against specific proteins in a food and *food intolerance*, which not involves the immune system (Bruijnzeel-Koomen et al., 1995). The food intolerances are classified into enzymatic, due to deficiencies of enzymes in food metabolism, and pharmacological due to substance normally present in some food, such as vasoactive amines, histamine and tyramine, to which some individuals are abnormally reactive.

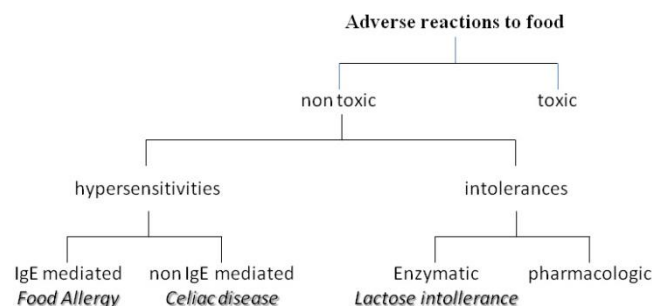


Figure 1. Classification of adverse reaction to foods.

In foods the specific antigens that elicit the reaction are foreign protein molecules (Sampson 1999). Only a few of the many proteins in nature are actually able to elicit immunological reactions in susceptible individuals (Taylor 1996).

In the sensitization phase of the *food allergy* also referred to as *type I allergy* or *sensitizing elicitor*, the first time the susceptible individual is exposed to the allergen the immune system recognizes the protein as harmful and responds by creating specific antibodies (IgE) against that allergen, which bind with high- specificity IgE receptors (FcεI) on the surface of mast cells, basophiles, Langerhans cells and monocytes of atopic subjects. Repeated exposures to the same food protein activate the binding to the specific IgE antibodies attached on mast cells in various tissues and basophiles in the blood activating these mediator cells. This elicitation causes the release of the inflammatory molecules, such as histamine, leukotrienes and prostaglandins (Taylor and Hefle 2001).

Although any food protein can potentially provoke an immune reaction, relatively few food proteins are responsible for the vast majority of significant food - induced allergic reactions (Boye et al., 2010). Usually less than 2% of the dietary proteins are absorbed in an immunologically intact form. When intact proteins do cross the gut barrier, the immune system physiologically induces oral tolerance, since the food allergy is a transient or permanent failure in development, or a breakdown of the functionality of the oral tolerance (Chehade et al., 2005).

Epitopes are the antigenic determinants of allergens. The severity of the clinical symptoms is strictly related to the structure of the epitope(s), which are classified in linear and conformational ones (Figure 2).

The specific effects of the food allergic reaction depend on the time and the dose of the offending food and may range from mild to annoying to severe and life-threatening symptoms (Lemke and Taylor 1994). IgE- mediated reactions can start within minutes to 1 hour (rarely past 2 hours) of exposure. In the 95% of the reactions, symptoms appeared in the first 20 min (Sicherer et al., 1998).

The "conformational IgE epitopes", typical of "type 2" foods allergens, involve non-sequential amino acids and bind IgE because of a higher order structural arrangement. The role of conformational IgE-binding epitopes is relevant to the etiology of aeroallergen-mediated allergic reactions. They are heat labile and the rapid unfolding of the three dimensional structure, that occurs during heat process, may reduce or completely annulled the IgE recognition. The

role of conformational epitopes is relevant for the etiology of aeroallergens-mediated allergic reactions. These epitopes are responsible for the so-called Oral Allergic Syndrome (OAS), a condition in which symptoms generally appears within 5 to 15 minutes following food ingestion and are confined to the oral and respiratory tract, including hives, itching and swelling and for this reason the stability to digestion is a not a critical factor for this epitope. Approximately 15–20% of the population in developed countries are allergic to pollen, and between 50 and 93% of birch pollen–allergic patients have IgE-mediated reactions to pollen-related foods. The majority of these reactions is caused by conformational epitopes of four distinct cross-reactive structures homologues to the birch pollen Bet v 1, the major birch pollen related allergen, which occur in pollens of several tree species (e.g., apples, stone fruits, celery, carrot, nuts, soybeans) (Vieths, et al., 2002). The access of food allergens cross-reactive with aeroallergens may occurs at oral or nasal mucosa level, the stability to gastrointestinal proteolysis is not request.

The fact that true food allergens undergo proteolysis in the gastrointestinal track before they may induce an IgE sensitization are mainly “*type I*” allergens, characterized by sequential or “linear IgE epitopes”. These epitopes are highly resistant to heat treatment and enzymatic digestion, thus preserving their capacity to elicit severe reactions with symptoms that include hypotension and hypoxia: confusion, collapse, unconsciousness, and incontinence. Since food are ingested, moderate reactions evolve the gastrointestinal tract: reflux, colic, abdominal pain, nausea, vomiting, diarrhea. Coetaneous symptoms such as flushing, dermatitis, urticaria, and angioedema are also present in most anaphylactic reactions.

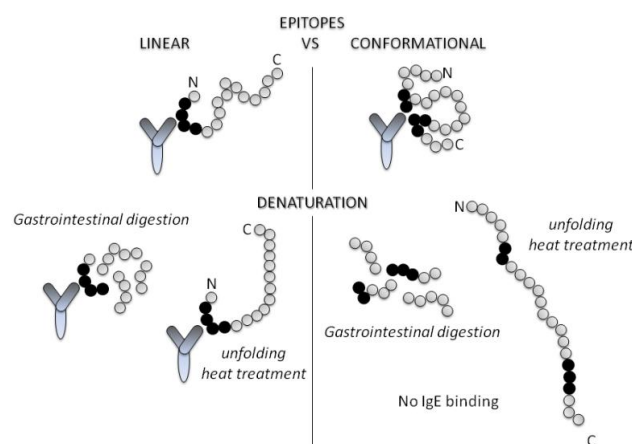


Figure 2. Schematic representation of linear and conformational epitopes. Linear epitopes after unfolding induced by heat treatment or digestion by gastrointestinal enzymes are still recognized by immunoglobulins whilst conformational epitopes are completely degradates.

Allergic reaction to foods are diagnosed by identifying the underlying immunochemical mechanism and identifying the specific serum IgE antibody determinants (Skin Prick Test, *in vitro* IgE assays) and establishing a reliable cause-effect relationship between ingestion of the suspected food and clinical manifestation reported by the patient (oral challenge) (Crespo et al., 2006).

Skin Prick test (SPT) and *in vitro* IgE assays are usually the most common test used in screening for specific IgE to foods. These assays are very useful in the initial evaluation of the allergic patience. *In vitro* tests are recommended in patients with extensive skin disease. The **skin prick test** consists of a tiny drop of solution containing the food proteins extract or the pure/recombinant suspected allergen slightly scratched with a needle on the surface of the skin. The positive reaction, which may occurs after few minutes, suggest the possible relation between food and symptoms. The diagnosis of food allergy must be confirmed by oral challenge. A negative response to skin prick testing usually indicates that the patient is not sensitive to that allergen. Several cases of low sensitivity to the SPT correlated to positive symptoms after food oral challenge have been largely documented. Allergen extracts for skin prick testing (SPT) are available from several manufacturers, are not standardized and may frequently miss of less abundant and labile allergens (Skamstrup Hansen et al., 2001). The diagnostic accuracy of both SPT and *in vitro* tests, in fact, is straight depend on the quality of the food and the efficiency of proteins extraction techniques, the susceptibility to protein modification and the variation in abundance of individual allergens in raw materials. To overcome the poor specificity of diagnostic tests and improve the sensitivity, specificity and reproducibility, in the recent years has been suggested the addiction to protein extract of purified and or recombinant allergens, for instance in hazelnut allergic diagnostic assay the addition of the thermolabile Cor a 1.04, homologues food allergen of the aeroallergen Bet v 1 (Andersson et al., 2007). In this contest the understanding the entire panel of allergic determinants of foodstuff is fundamental (Fernández-Rivas et al., 2004).



## 1.2 Allergy to Tree Nuts and Edible Seeds: Hazelnut allergy.

Tree nuts, among which are hazelnuts (*Corylus avellana*), most frequently cause serious and life-threatening symptoms in sensitive subjects. On the other hand the consumption of nuts can cause many health benefits on the outcome of coronary disease and cholesterol serum levels (Crespo et al., 2006). The 70% of allergic reactions to nuts occur after the first exposure.

Hazelnuts are the kernels of the hazel (*Corylus*) tree and the multiple varieties of hazel trees all produce edible nuts, but the common hazel (*Corylus avellana*) is the most extensively grown for its nuts (Platteau et al., 2011).

Hazelnut production in Italy is highly concentrated in few regions and is characterized by a very high number of varieties grown, sometimes even within the same area. Among the most common varieties in Campania are the Tonda of Giffoni, the Mortarella, and the Lunga of San Giovanni; in Lazio we find the Tonda gentile Romana and in Piemonte the Tonda Gentile of Langhe. The Tonda of Giffoni and the Tonda Gentile of Langhe, known also as Piedimonte have been awarded of the IGP protected geographical indication which defines the geographical area where these cultivars can be grown. Hazelnuts are one of the most nutritious nuts, high in energy and kilojoules, with a protein content of about 12%. They are also a good source of energy, with a fat content of about 60%, and an excellent source of carbohydrates, among which half are dietary fibre. Hazelnuts also contain minerals (Ca, Mg, P, K), vitamin E, B vitamins, and antioxidants. Hazelnuts contain multiple allergenic proteins, which can induce a life-long condition allergic reaction with symptoms that can vary from mild to severe, including anaphylaxis. Hazelnuts are widely used in the food industry, especially in confectionery food (chocolates, nougat cookies, pralines, chopped nuts, nut spreads, breakfast cereals), where they are used raw, roasted, or in a processed formulation (e.g., praline paste and hazelnut oil).

The prevalence in European country of hazelnut allergy range between 0.1–0.5% (Koppelman et al. 1999) whilst the global prevalence is estimated to be between 0.1 and 4% (Zuidmeer et al., 2008). Unfortunately, an exact evaluation of the prevalence of hazelnut allergy is hindered at least in part by the lack of availability of standardised allergens for clinical use (Andersson et al., 2007).

The relative allergenicity of a food protein is defined on the base of the percentage of individuals having IgE for that specific allergen. Major allergens are proteins for which more than 50% of the allergic patients studied have specific IgE (Fu 2002). Over the years, at least nine proteins have been reported as potential hazelnut allergens. The predominance of specific allergens appears to be strictly related to both the geographical origin (Pastorello et al., 2002) and the age of the allergic subjects (Verweij et al., 2012).

In birch-endemic regions of Northern Europe, hazelnut allergy has been primarily associated with the Cor a 1.04 for cross-reactivity with the aeroallergens Bet v 1 from *Betula verrucosa* in adulthood, as well as to a minor 14 kDa hazelnut allergen, Cor a 2 related to the birch pollen profilin Bet v 2 (Ebner et al., 1995). The Cor a 1 is a monomeric protein of about 17.4 kDa and its isoforms are expressed in at least four sub-forms which are 97-99% identical to each other but share only 63 and 71% identity with hazel pollen isoforms (Lüttkopf et al., 2002). The pollen-related hazelnut allergy usually induces mild oral allergy syndromes (OAS). Cor a 1.04 is a thermolabile allergen and easily undergoes pepsinolysis digestion. The IgE-binding significantly is reduced by a factor of 100 after heat treatment and the activity is almost completely absent after roasting (140°C), since the native three-dimensional structure is rapidly destroyed (Worm et al., 2009; Hansen et al., 2003). All the patients affected by OAS, independently to the ages, are sensitized to Bet v 1 and the homologues Cor a 1.04 and Cor a 1.0101 (De Knop et al., 2011).

The clinical symptoms of non-pollen related allergy to hazelnut can be severe and systemic and are frequently associated with the presence of linear epitopes.

Globulins typically deposited in high quantities in the maturing nut and are classified on the base of the sedimentation coefficient, which may vary between 12S and 7S. The 11/12S legumins, belonging to the *Cupin* family, are trimers and/or hexamer of about 50 kDa subunits held together by non-covalent interactions. They are synthesized as single polypeptides, which that undergoes post-translational cleavage at an Asn-Gly bond by an asparaginyl endopeptidase that split the protein into an acidic (Mr ~ 30–40 kDa) and a basic (Mr ~ 20 kDa) polypeptide chains linked by a single intermolecular disulphide bond. 11S globulins of tree nuts and legumes display from ~8 to 15 linear epitope-bearing peptide regions that are scattered throughout the length of the acidic and basic subunits (Robotham et al., 2009).

The 8/7S *vicilin* are typically trimeric proteins of Mr about 150/190 kDa, with subunits of about 50 kDa. These proteins contain no disulphide bonds (Mills et al., 2004).

The main non-pollen-associated allergen in the United States appears to be the 11S globulin-like protein, Cor a 9 allergen, that is composed of a 30–40 kDa acidic subunit and a 20–25 kDa basic subunit linked via an inter-chain disulphide bridge (Beyer et al., 2002). In particular, only the acidic chain is immunoreactive. The conformational analysis of the surface exposed IgE binding epitopes revealed that Cor a 9 exhibit some homology with other tree nut allergens the cashews allergen Ana o 2, the peanut Ara h 2 allergen and the soybean glycine (Barre et al., 2007).

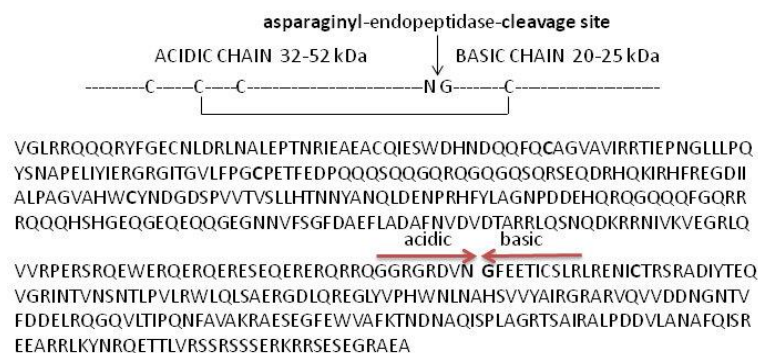


Figure 3. Amino acid sequence of 11S globulin-like proteins from *Corylus avellana*. Schematic diagram illustrating the subunit structure and processing of the 11S.

Recent clinical trials, carried out using immunoCAP assays, demonstrated the extensive sensitization in children recruited in birch endemic region (7-9 years old), to the nCor a 9 and the very low immunorecognition for the expected rCor a 1 and rCor a 8 (Verweij et al. 2001). A similar study showed in young subjects an additional less extended immunoreactivity to the 48-kDa glycoprotein, Cor a 11 allergen (Verweij et al., 2012).

Protein glycosylation (i.e., oligosaccharides covalently attached to proteins) is estimated to be the most common post-translational modification, occurring in approximately 50% of proteins (Apweiler et al., 1999). Plant oligosaccharides are generally classified in two general groups, high-mannose (e.g., Man(5-9)GlcNAc2) and complex (e.g., core structure of Man3GlcNAc2 with addition of other sugars). The characteristic addition in plants and invertebrates of  $\alpha$ (1,2)-xylose residue to the mannose and/or, to a lesser extent,  $\beta$  (1,3)-fucose residue to the proximal GlcNAc (Garcia-Casado et al., 1996; van Ree et al., 2000) play the key role in the formation of conformational IgE binding epitopes (glycol-epitopes) in mammalian. Plant glycoproteins share a limited set of conserved glycan structure that account for the cross-reactivity between vicilin allergen of nuts. Even though about 20% or more of allergic patients generate specific anti-glycan IgE, which is often accompanied by IgG (Altmann et al., 2007), their effective clinical relevance in eliciting an allergic reaction is controversial. Examples of IgE binding glycans are the Api m 1 (Tretter et al., 1993) from honeybee (*Apis mellifera*) venom, Cup a 1 (Alisi et al., 2001) from cypress (*Cupressus arizonica*) pollen, Lyc e 2 (Foetisch et al., 2003), Api g 5 (Bublin 2003) from celery (*Apium graveolens*), and Ara h 1 (Van Ree et al., 2000) from peanut. The actual allergenic potential of glycoproteins has been only confirmed for the Lyc e 2 (Foetisch et al., 2003), the Api g 5 (Bublin 2003).

The 48 kDa glycoprotein, Cor a 11 allergen, sequence contains potential glycosylation sites. The characterization at molecular level of the N-glycan structures of the purified natural hazelnut vicilin demonstrated that only one is actually glycosylated and, more in deep, that 74% of the glycans are MMX-type and 3% MMXF-type (Lauer et al., 2004).

```

SSEESYGKEQEENPYVFQDEHFESRVKTEEGRVQVLENFTKRSRLLSGIEFRLAILEANPH
TFISPAHFDAELVLFVAKGRATITMVREEKRESFNVHGDHRIIPAGTPVYMINRDENEKLF
VKILQPVSA PGHFEAFY GAGGEDPESFYRAFSWEVLEAALKVRREQLEKVFGEQSKGSIVK
ASREKIRALSQHEEGPPIWPFGGESSGPINLLHKHPSQSNQFGRLYEAHPDDHKQLQDL
DLMVSFANITKGS MAGPYNSRATKISVVVEGEGFFEMACPHLSSSSGSYQKISARLRRV
VFVAPAGHPVAVIASQNNLQVLCFEVNAHGNSRFPLAGKGNIVNEFERDAKELAFNLPS
REVERIFKNQDQAFFFPGPKNQOEGRGGRFAFE
  
```

Figure 4. Amino acid sequences of 48 kDa- glycoprotein from *Corylus avellana*. The two possible N-glycosylation sites (NXT) are highlighted with red large letters.

Although Muller et al., 2000 demonstrated that hazelnut allergic patients had cross-linking IgE antibodies binding to the protein backbone and not to carbohydrate structures, comparing the immunoreactivity of glycosylated and deglycosylated forms of the Cor a 11, on the other hand, since N-glycans have binding affinity for aromatic amino acid residues which protects proteins from protease digestion, is not entirely possible to rule out the involvement of N-glycans at least in conferring stability against proteolytic degradation (Nishiyama et al., 2000).

In areas without birches (the Mediterranean area), severe life-threatening allergies in adults are associated with sensitization to the *non-specific* Lipid Transfer Protein (ns-LTP), allergen Cor a 8. The ns-LTP can be integrated in the huge superfamily of prolamines. The primary structure of the mature LTP comprises a unique polypeptide chain of 90-95 amino acid residues. Among this, there are eight strictly conserved cysteine residues that form 4 intra-chain disulphide bridges (Mills et al., 2004).



Figure 5. Amino acid sequence and cysteine pairing of ns-LTP from *Corylus avellana*.

The secondary structure is composed of 4  $\alpha$ -helices and a long carboxyl terminal tail that is devoid of a defined secondary structure. The three-dimensional structure reveals an internal flexible hydrophobic cavity in a calyx like that can accommodate a variety of lipids.

This protein are widespread in plant kingdom and because of their very conserved sequence regions across the species, ns-LTPs are the causative agents of documented cross reactive allergy to a large spectrum of botanically unrelated plant-derived foods (Wijesinha-Bettoni et al., 2010). The characteristic structure of the prolamine family makes the protein very tough and resistant to both heat treatment and proteolytic digestion. This seems to be the key parameters relating to their allergenicity largely associated to severe anaphylactic reactions (Schocker et al., 2004). Clinical- epidemiology studies define the Cor a 8 as the major allergic determinant for the 5.4% of hazelnut allergic subjects in Italy.

The 2S albumins are typically heterodimeric water-soluble seed storage proteins, which share a characteristic four helix and the four disulfide bridges structure with the ns-LTP. As well as the 11S globulin is cleavage by a post-translational processing of the precursor protein into a large and a small subunit bound together *via* disulphide bridges (Garino et al., 2010). 2S albumins are considered major allergens in walnut, Brazil nuts, oilseed rape, castor beans and mustard seed (Moreno and Clemente 2008). As well as the Cor a 9, Cor a 8 and Cor a 11 also the 2S albumin, Cor a 14 allergen, have been described as very dangerous allergen responsible for anaphylaxis since the structure tend to be relatively resistant to denaturation and proteolysis.

Two oleosin isoforms (Cor a 12 and Cor a 13) were identified as allergens in hazelnut (Akkerdaas et al., 2006).

The complex patterns of IgE reactivity may result in poor diagnostic and prognostic accuracy (Akkerdaas et al., 2003a and b). Because the clinical symptoms of hazelnut allergy seem to be associated with the nature of the allergens, the component-resolved “molecular approach” aimed at the precise identification of allergen(s) is expected to greatly improve both the diagnosis and treatment of food allergies.

### 1.3 “Allergenomics”

Advanced proteomic and bioinformatics strategies, together with *in vivo* and *in vitro*, and/or clinical assays, are applied in food studies for a more detailed and comprehensive analysis of putative food allergens at molecular level. This approach has been termed “allergenomics” (Akagawa M. et al., 2007).

Mass spectrometry techniques are the fundamental tool in food allergenomics. The classical “bottom-up” approaches that identified and characterize digested proteins and the “top-down” approach that characterize intact proteins, based on “soft” ionization sources such as Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS), and Electrospray ionization (ESI) are extensively applied for quali-quantitative analysis in proteomics. The main difference between the two ionization techniques is that MALDI is employed on samples in a solid surface whereas ESI is employed on samples in a liquid state. Moreover MALDI is an off line process whilst ESI has the ability to interface liquid chromatography to mass spectrometry analysis. The extreme accuracy of ESI-MS for measuring protein molecular weight is complemented by the capability of MALDI-MS to analyze proteins with a mass greater than 100 kDa or polypeptides in complex mixtures (Mamone et al., 2009). ESI and MALDI ionization sources may be combined with a number of analyzers which separate ionized molecules according to their  $m/z$  ratio. The top four routinely mass analyzer used in proteomics are: quadrupole (Q), ion-trap (quadrupole ion trap, QIT; linear ion trap, LIT or LTQ), time-of-flight (ToF) and Fourier-transform ion cyclotron resonance (FTICR) mass analyzers. They strongly differ in both the physical principles of ion separation and the analytical performances. Multi-stage and ‘hybrid’ instruments have been designed to combine the capabilities of different mass analyzers including the Q-q-Q (triple quadrupole), Q-q-LIT, Q-TOF, TOF-TOF, and LTQ-FTICR. Quadrupole TOF (Q-TOF) and tandem TOF (TOF<sub>2</sub>) instruments are most suitable for tackling the challenges of protein and proteome analysis, due to their capability in performing both quantitative analysis and identification of PTMs and in the investigation of food allergens modifications occurring during food transformations. Q-TOF offers good sensitivity, resolution and mass accuracy and allows fragmentation of MALDI-generated precursor ions. In addition, Q-TOF can be used interchangeably with an Electrospray ionization source (Monaci and Visconti 2009).

Mass spectrometry techniques may provide the molecular mass of protein/derived peptides (MS1) and structural additional details such as peptides sequence and post translational modifications (PTMs) from tandem mass spectra (MS/MS) obtained either by post-source decay (PSD) or, especially in the hybrid arrays, collision induced dissociation (CID) making the search highly specific and discriminating (Mann et al., 2001). The performance of mass analysis varies in sensitivity, resolution, mass accuracy and ability to produce high quality MS and MS/MS spectra.

Identification of allergens is less straightforward when the genome of the matrix of interest or of related organism is not completely sequenced. In this case the generation of *de novo* amino acid sequence information by tandem mass spectrometry and the sequencing is very helpful in identifying new allergens.

The structural characterization of post translational modifications, among which the glycosylation, is particularly relevant for food glycoallergens, since they play a key-role in the formation of IgE glycol-epitopes. The mass spectrometry characterization of glycoproteins shows to have several intrinsic drawbacks, such as the significant suppression of glycopeptides ions when are analyzed in complex mixture of peptides. The so called Hydrophilic Interaction Liquid Chromatography, HILIC, characterized by the use of a hydrophilic stationary phase and a hydrophobic organic mobile phase, has been demonstrated to be a successfully technique for specific separation and enrichment of glycopeptides from complex digested mixture. The order of elution is reversed relative to reversed-phase chromatography (RP), with hydrophilic compounds being retained longer than hydrophobic compounds (Boersema et al., 2009). By this way the enriched glycopeptides can be characterized separately and glycans and glycosylation site can be performed by MALDI or ESI MS analysis after enzymatic hydrolytic cleavage.

The optimization of sample extraction and preparation is critical steps for a high-quality analysis of proteomes (Natarajan et al., 2009). It is dependent of food matrix quality and the target metabolites of interest. In fact, applying different techniques of extraction to the same matrix, the quality of total proteome extract may vary widely in both reproducibility and completeness.

Surface-enhanced laser desorption/ionization (SELDI) is an ionization method in mass spectrometry that is used for the analysis of protein in combination with time-of-flight mass spectrometers. The protein mixture is spotted on a surface modified with a chemical functionality. Some proteins in the sample bind to the surface, while the others are removed by washing. SELDI is a useful system for rapid separation, detection and analysis of proteins at femtomole levels directly from biological samples. The main advantage is the speed of detection that is reduced to few hours and the coverage of a broad region of the proteome, small samples request and the combination of discovery biomarker (Kvasnicka 2003).

The new generation of hybrid (Q/TOF, Orbitrap) instrument which combined a linear ion trap with an Orbitrap analyzer has become one of the standard mass spectrometers in protein allergens detection once the peptide markers are identified (Monaci et al., 2010, 2011a). Monaci et al 2001b, demonstrated that application of Orbitrap analyzer, that allowed extremely high mass accuracy and resolution, provided a fast preliminary identification of four previously proposed peptide markers of caseins using only accurate values of the  $m/z$  of their ions with a LOD range between 0.15-0.7 ppm in wine, depending on the peptide selected.

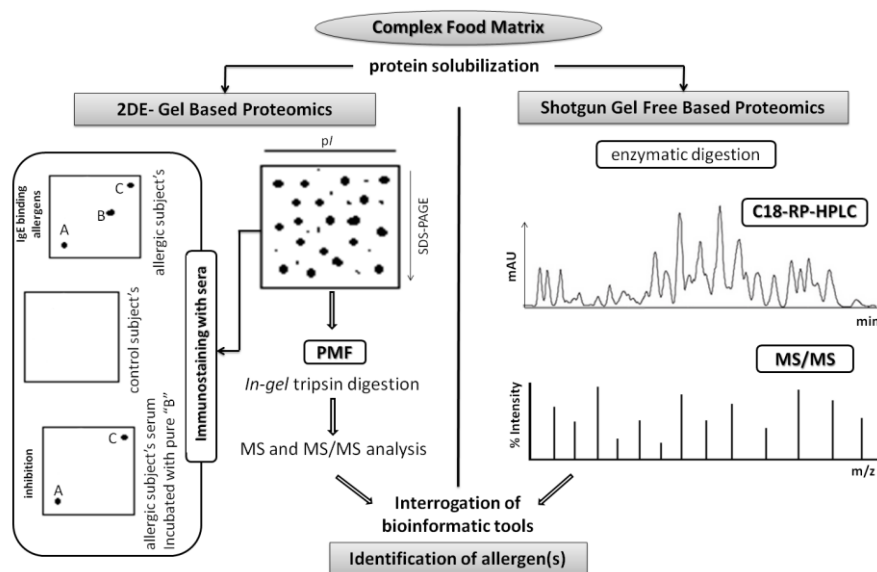
The mass spectrometry analysis of complex mixture of derived protein moieties after digestion lacks the probability to detect and confidentially identify at least one constituent peptide, due to the mutual ion suppression effect. No single analytical methodology or platform is applicable to detect, quantify and identify all metabolites in a certain sample. Mass spectrometry analyses are usually coupled with high resolution separation techniques such as chromatography and electrophoresis.

The **gel-based proteomic techniques** (figure 6) comprehend mono and two-dimensional gel electrophoresis separation before identification of proteins by mass spectrometry. This strategy allows the separation of thousands of proteins on a single step, according to their  $pI$  (isoelectric focusing, IEF) and sub-sequentially to their molecular weight (SDS-PAGE). This technique is an extremely powerful tool for separating proteins of similar molecular mass, but, despite the high resolution offered, it has significant drawbacks. such as low reproducibility, low sensitivity, gel-to-gel variability, low dynamic range for quantification and poor detection of very hydrophobic and alkaline proteins (Beranova-Giorgianni et al., 2003; Monaci and Visconti 2009). Proteins visualized by different staining techniques, such as Comassie, silver, Sypro, can be excised from the gel, *in situ* digested with a protease and identified by Peptide Mass Fingerprinting (PMF). This identification technique consists in the generation of a set of peptides that are unique for each protein and determinate peptide masses and or sequence by tandem mass spectrometry analysis (MALDI-Tof/Tof, ESI-MS/MS) are used as a fingerprint for comparison with theoretical expected tryptic peptides for each entry in online proteomic and genomic databases.

To examine the immunological responses against food proteome, the 1D and 2D electrophoresis can be combined with immunoblotting analysis, where proteins are stained with sera from allergic subjects as source of immunoglobulins. In dot-immunoblotting proteins and peptides are spotted onto membrane strips whilst in western blotting analysis proteins are previously separated by electrophoresis according, to their molecular mass and, afterwards, are transferred onto a membrane. Sera from allergic subjects are used as specific immunoglobulin source. The detection is achieved by incubation with enzyme-labeled antibodies which bind to the target antigens. The immunodetection is usually carried out using a secondary antibody, which is a specific anti-human antibody. The intensity of the spots is proportional to the amount of antigen. By this way is possible the identification and the characterization the specific allergic determinant(s), for one or a pool of sera over thousands of proteins simultaneously. The validation of the effective immunoreactive potential of a protein can be usefully validate by inhibition assays, in which the food proteome is blotted with sera from allergic subjects pre-incubated with the purified candidate allergen.

Capillary electrophoresis, also known as capillary zone electrophoresis (CZE), can be used to separate ionic species by their charge and frictional forces and hydrodynamic radius. CE is a hopeful tool for on-line concentration of low abundance protein samples for proteomic research. An enhancement factor of more than 100 times was achieved to the conventional pressure injection method (Kim et al., 2009).

Electrophoresis separation pitfalls in mass spectrometry identification of less abundant proteins and may lead to false positive results in mass spectrometry identification because are frequently identified by a single peptide sequence, with low score and low significance. The recent introduction of fluorescence-based differential in-gel electrophoresis (DIGE) has allowed more accurate, more sensitive, quantitative results. To enhance analysis, pre-fractionation of proteins from a sample is often employed prior to 2D-PAGE. In DIGE, two pools of proteins are labeled with 1-(5-carboxypentyl)-1'-propylindocarbocyanine halide (Cy3) N-hydroxy-succinimidyl ester and 1-(5-carboxypentyl)-1'-methylindocarbocyanine halide (Cy5) N-hydroxysuccinimidyl ester fluorescent dyes, respectively. The labeled proteins are mixed and separated in the same 2D gel. This technique allowed removing gel-to-gel variability and makes possible a higher accuracy of relative quantitation (Keeler et al., 2007).



**Figure 6.** 2D-based approach for allergens identification and characterization. Proteins extract from food matrix is separated by 2D electrophoresis and immunoblotted against sera of allergic subjects. Immunoreactive proteins are identified by peptide mass fingerprinting. Confirmation of antigenic properties can be confirmed by inhibition blotting assay, where sera of allergic subjects is pre-incubated with pure putative allergen. New allergens can be detected and identified by de novo peptide sequencing. In comparison the shotgun gel-free approach where crude protein extract undergoes enzymatic digestion (typically is used trypsin) and directly analyzed by tandem mass liquid chromatography. The interrogation of bioinformatic tools allowed the identification of already indexed allergens.

An important challenge in proteomic studies is the wide difference in concentration from the most- to the least-abundant proteins (Herrero et al., 2011). The extensive predominance of globulins, in seeds storage proteome, defies the identification of less-abundance components such as profilin and prolamine. To reduce the complexity of the proteome and improve the quality of low abundant proteins analysis is possible to applied several chromatographic techniques for fractionation, depletion and enrichment.

Liquid-phase separation is becoming more common for protein separation in proteomic studies, since offers superior dynamic range than 2D-PAGE but is limited by reduced resolution. New generation **gel free-based strategy** (Figure 6), referred to as “**shotgun**” proteomics, are based on a multidimensional liquid chromatographic (LC) separation prior to ionization and tandem mass spectrometry (MS/MS) analysis mass spectrometry analysis (Lu et al., 2009; Yattes III et al., 2004). By this technique is possible to analyse the complex trypsinized mixture of the whole protein extract preventing the possible pitfalls of the 2-dimensional (2D) electrophoresis separation. Proteomics studies of complex peptides mixtures result in thousands to millions of MS/MS spectra. The multiplication of protein-specific peptide sequences enhances the probability of detecting and confidentially identifying at least one peptide of each parent protein. The interpretation requests sophisticated algorithms. Data are directly identified using powerful database searching tools such as Protein Prospector, MASCOT, and SEQUEST in combination with continually update database such as Swissprot and NCBI (Johnson et al., 2005). The support of effective bioinformatic tools for data interpretation is essential to deduce useful information from the analytical process of a huge amount of spectra at the same time.

**Multidimensional identification (MultiID) technology** is now widely implemented in proteomic analysis, providing more separation power, extended measured dynamic concentration range, good resolution and peak capacity. Two-dimensional columns switching liquid chromatography consist in two columns coupled with a switching valve. The separation of peptides mixtures by LC7LC methods has been performed using several combinations including size-exclusion chromatography (SEC) with reversed-phase LC (RPLC) (Opiteck and Jorgenson, 1997), RPLC with Capillary Electrophoresis (Haselberg et al., 2007), strong cation-exchange chromatography (SCX) with RP-HPLC (Link et al., 1999), SCX with affinity chromatography (AC) to select specifically biotinylated peptides (Gygi et al., 2002) and isoelectric focusing (IEF) with RPLC (Cargile et al., 2004). MultiID can be also applied in peptide separation by ultra-high-pressure LC (UHPLC) and anion-and-cation mixed-bed ion-exchange techniques (Han et al., 2008). In the most of shotgun methods the second dimension is performed by RPLC since the mobile phase is compatible with mass spectrometer.

Mass spectrometry is not intrinsically quantitative because of differences in the ionization efficiency and/or detectability of the peptides and proteins, due to the molecular specific physiochemical properties. **MRM (Multiple Reaction Monitoring) mass spectrometry** is a highly sensitive and selective method for quantitative analysis of biomarker abundances candidate, both peptides and/or proteins, so called “proteotypic-peptides”, in complex biological samples. These biomarkers are previously selected by explorative analysis: pure *in silico* design from sequence databases, design from available LC-MS/MS proteomic survey data, and comprehensive MRM testing of all of the candidate peptides of a protein. Once the biomarker peptides are selected they become the base for the synthesis of stable isotope-<sup>13</sup>C and/or <sup>15</sup>N- labeled standard (SIS). In this way the inherent instability of Electrospray ionization is overcome by the addition of these isotope peptides into the sample in a known quantity provides accurate and precise quantification. The isotopic marker introduces little chromatographic shift in reversed-phase chromatography so that they coelute with the target peptide and are chemically identical to the target peptide, except for the mass difference. **AQUA (Absolute QUantification)** peptides are synthetic commercial copies of proteotypic-peptides used as internal standard for absolute quantification. The mass spectrometer is set up to scan for ideal transition ions (precursor-to-product) of target peptides, both labelled and unlabeled in the case of AQUA analysis. Ion current signals are plotted over time and integrated to calculate peak areas. Peak areas for AQUA and native peptides are compared to calculate absolute quantities of native peptide in the sample.

Using the MRM-MS based strategy, ten target allergens have been quantified in commercial soybean cultivars and the differential expression of five allergens (glycinin G3, glycinin g4,  $\beta$  conglycinin and Kunitz trypsin inhibitor 1) has been determined across 20 varieties (Houston et al., 2011), suggesting expression differences among the varieties. The same approach resulted resolute in monitoring hordein subtypes, harmful for celiac subjects, in 60 beer samples (Colgrave et al., 2012).

### 1.3.1 Identification of allergens in transformed products.

Avoidance of allergic food is the only way for allergic people to prevent the occurrence of food allergy. The ability to identify foods containing allergens is therefore vital to these people (Fu, 2002).

Proteins derived from allergic foods, may be accidentally present in processed food as the only result of cross-contact during processing and handling. Cross reaction of food with allergens has been shown to lead to allergic reaction in consumers on several occasions (Gern et al. 1991). The contamination can occur when multiple foods are produced in the same facility or on the same processing line and trace of food allergen becomes incorporated to another food items often by way of contact with unwashed surfaces of the production machinery, dusty processing environments. Food manufacturing workers themselves can be triggered by food derived protein allergens by respiratory exposure to or coetaneous exposure to specific food products (Lehrer and O Neil, 1992).

Considering that also traces of allergens can be harmful in some allergic subjects, the European Commission Directive 2007/68/EC proposed several directives to discipline labeling of potential sources of allergens in foodstuffs (European Parliament and Council, 2007). Little is known about the so called threshold doses, i.e. the minimum amount of an allergenic food which is able to cause an allergic reaction (Bignardi et al., 2010). Taylor and Nordlee et al., 1995 postulated that immunoassays for the detection of food allergens should have detection limits of at least 10 ppm since allergic individuals can react to traces of this order of magnitude.

Regardless of the labelling, several cases of contamination of foods with hazelnuts are reported (Rapid Alert System for Food and Feed (RASFF) 2011), which is leading to an important health problem (Cucu et al., 2012).

The most common routinely test used by industry and enforcement agencies for allergen semi-quantitative detection in foods include **enzyme-linked immunosorbent assay (ELISA)**, dipstick or Western blotting and PCR-based analysis. ELISAs are most frequently performed in 96-well microplates or in 8-well strips. The *sandwich ELISA* is the most common type of immunoassay performed. This format involves an immobilized capture antibody on the microplate wells. When the food sample is loaded into the well the antibody-analyte binding occurs. A second specific antibody labelled with an enzyme is added and also binds to the analyte, forming a "sandwich". The development is carried out using a specific substrate that reacts with the enzyme and produces a coloured product. The absorption is directly proportional to the concentration of the analyte. Typical limits of detections (LODs) of the ELISA tests fall in the range of the low ppm (1–5 ppm). The *competitive ELISA* involves immobilized antigens

bound to the solid phase. When the epitope is absent in the sample assayed, the enzyme-labelled antibody shows maximal binding to the solid phase bound antigen, resulting in high absorption of the coloured product formed. Binding of the enzyme-labelled antibody is inhibited by increasing amounts of antigen. ELISA kits are commercially available and are used for rapid semi-quantitative screening. Haas-Lauterbach et al., 2012 recently demonstrated that the competitive ELISA provided significantly higher prolamin concentrations, compared to the sandwich ELISA based on the same antibody, which is only suitable for the detection of intact prolamins when analyzed beer samples and a hydrolyzed of wheat product. Koppelman et al., 1999 demonstrated that the sandwich ELISA, constructed with immunopurified antibodies obtained from rabbits sensitized with hazelnut proteins was able to detect and quantify traces of physiologically relevant hazelnut allergens as low as 1 ppm. The main limits of these techniques are that provide only a static snapshot of the allergens since miss of two fundamental informations: the level of expression of the proteins and the posttranslational modification. Frequently the epitopes used in the kit are aspecifics and for this reason cross-reactivity with the matrix components can results in false positives. Sandwich immunoassay demonstrated a detection sensibility of hazelnut protein between 0.001 and 10% (Ben Rejeb et al., 2003).

Since hazelnut belong to the same botanical order of walnut and pecan, cross-reactive with them can occur. In fact, the commercial hazelnut ELISA test, that is based on polyclonal antibody raised against a whole hazelnut protein extract showed cross reactivity to different nuts extract among which walnut (0.001 – 0.036 %) (Ehlert et al., 2009). When the technique was applied to complex food matrixes, the yield of recovery was of about 67-132%. Koppelman et al. 1999 described a similar method able to detect as little as 5 ng/mL of hazelnut proteins in solution, corresponding to 1 µg/g in food products. Immuno-electrodiffusion allowed the quantification of 1–15% hazelnut in food products (Klein et al., 1985).

DNA based methods consist in the PCR amplification of DNA fragments specific for the allergic food. Detecting DNA from allergenic sources is just at the beginning of its development. Only very few applications of PCR-reactions for the detection of allergens, namely hazelnut and wheat, have been published (Koeppel et al. 1998). The main limit of these techniques is that the presence of DNA of a food component in a food product does not guarantee the presence of the allergens and this may affect false positive results. Moreover, the stability and the sensitivity of immunochemical and DNA based methods strictly depend on thermal and technological treatments. A new PCR-based approach is the simultaneous detection of DNA from various food allergens by **ligation-dependent probe (LDP)**. Ligation dependent PCR is a technique originally used for detection of nucleic acid, which permits to be amplified multiple targets with only a single primer-pair. Each probe consists of two oligonucleotides which recognise adjacent target sites on the DNA. One probe oligonucleotide contains the sequence recognised by the forward primer, the other the sequence recognised by the reverse primer. Only when both probe oligonucleotides are hybridised to their respective targets, can they be ligated into a complete probe. The ligation of bipartite hybridization probes that bind to a target DNA derived from the food matrix under investigation (Iwoby et al., 2012). Ehlert et al., 2012 showed the sensitivity of this technique in simultaneous detection of trace amounts of hazelnut in chocolate and cookies with a LODs of 1000 mg/kg for peanut, hazelnut, pecan, macadamia when analyzed in mixture and 100 mg/kg for only hazelnut.

In the last years, increasing emphasis has been put on the development of confirmatory methods based on the use of **liquid chromatography–Electrospray-tandem mass spectrometry (LC–ESI-MS/MS)** techniques (Monaci et al., 2009; Picariello et al., 2012; Picariello et al., 2013; Shefcheck et al., 2006; Weber et al., 2006).

The **shotgun** proteomic technique takes advantage from the high sensitivity and specificity in detection of peptides than to large-sized proteins. Moreover expands the analytical dynamic range, enabling the detection of the less-abundance components and, in the case of foodstuff, the monitoring of “hidden allergens” in trace amounts. Ansari et al., 2011 described eight triptic peptides of three hazelnut allergens, Cor a 9, Cor a 11 and Cor a 8 with high intensity mass signals. Among this only one peptide of Cor a 9 was not considered a high-quality marker for LC/MS-MS monitoring of hidden hazelnut allergens because of the high homology of sequence with other 11S globulin like proteins. Bignardi et al., 2010 described the peptide QEWEER of the Cor a 9 as high-quality biomarker to detect by tandem mass spectrometry the presence of hazelnut hidden allergens at trace level in complex food matrix with a LODs ranging between 10 to 50 mg nut/kg matrix and a precision from 3 to 15%.

The modifications of proteins from raw material to finished foods are extremely relevant. The current tests may underestimate the detection of linear epitopes that could arise from proteolytic events such as fermentation in dairy products and beverages (Picariello et al., 2013).

The 11S globulins are highly thermostable and may unfold at temperature above 94°C, whilst the 7S have their thermal transition at 70-75°C. Since structural modifications are much more extensive when the content of water is high, typical dry heat treatments of nuts such as roasting do not reduce protein allergenicity (Hansen et al., 2003). The extensive treatment to microwave drastically reduces the immunoreactivity. The reduction of immunoreactive intensity is not evident when proteins are essayed *via* western-blotting analysis probably because the heat treatment affects mainly the conformational epitopes that are not anymore detectable under reducing conditions.

Moreover, hazelnuts proteins are widely used nowadays in food products containing significant amounts of sugars and fats (e.g. cookies, chocolate, breakfast cereals and nut spreads). During thermal treatment (roasting) and storage, hazelnut proteins, for instance might be glycosylated by interaction with reducing sugars (Maillard reaction) or oxidized



by interaction with lipids and carbonylation. These events might decrease allergenicity or conversely generate new-allergens, neo-epitopes not existing before (cross-linking, conjugation and deamidation) (Picariello et al., 2011). These modifications may lead the presence of hidden hazelnut in foods indicating that food products declared free of hazelnut allergens by the commercial kits could still affect the allergic consumers. Become fundamental the identification of stable allergen-derived peptides to use when are analysed complex matrixes. Cucu et al., 2012 described the peptides Gly395-Arg403 from Cor a 11 and Gln209-Arg217, Ile351-Arg363, Ala464-Arg478 and Val401-Arg417 from Cor a 9 as the most stable to Maillard reaction suggesting this peptides ad biomarkers quantitative confirmation of hidden allergenic hazelnut proteins. The allergenicity of barley *ns*-LTP1 is reduced when glycosylated through Maillard reactions during malting, especially when the water content of green malt decreases on heating (Jégou et al., 2000; Jégou et al., 2001).

### 1.3.2 Effect of gastrointestinal proteolysis on allergens.

Food allergens (or fragments) must cross intact the human gastrointestinal system, surviving to the acid and proteolytic environments, traslocate the gut barrier to reach the mucosal immune system and elicit the allergic reaction (Taylor et al., 1987). Stability to pepsin is for class I food allergens has been included in the Food and Agriculture Organization/World Health Organization decision-tree to asses safety of foods produced trough agricultural biotechnology (Astwood et al., 1996). These proteins mainly affect the gastrointestinal tract of young children and may account for severe anaphylactic reactions. The ability of the allergenic protein to trigger direct oral sensitization is modulated by gastric acidity (Knippels et al., 2003). Some food allergens demonstrated extensive and high resistance to digestion by pepsin when incubated under physiological condition in simulate gastric juice (SGF) (pH 3, 37 °C, 60 minutes) whilst all of non-allergen proteins were digested to low molecular weight peptides (<3000 kDa) within 15 seconds (Astwood et al., 1996). In contrast, Yagami et al., 2000 demonstrated that latex and vegetable food proteins were digested by the SGF within few minutes. Although some vegetables allergen were decomposed by the SGF within 8 minutes whilst other allergens, for instance kiwi allergens, were substantially degraded by the simulate intestinal fluid (SIF) within 16 hours.

A sub sequential pepsinolysis in artificial gastric fluid (2 h) followed by hydrolysis with pancreatic enzymes (45 min) resulted in reduced IgE-binding of digested hazelnut proteins. The EAST analysis with sera from hazelnut allergic individuals demonstrated a reduction of about 10% of IgE-binding potency of native protein extract (Vieths et al. 1999). Wigotzki et al. (2000 a) investigated the stability of hazelnut protein extracts against various enzymes. Peptic hydrolysis for 60 min induced only a slight decrease in IgE-binding (max. EAST inhibition appr. 65%). Even after 240 min of peptic hydrolysis two out seven sera from hazelnut allergic subjects showed IgE-binding in SDS-PAGE immunoblot. Maximum EAST inhibition was about 40% as compared to native hazelnut extract. In contrast, hydrolysis of hazelnut proteins with pancreatic enzymes significantly decreased the IgE-binding potential already after 30 min of treatment to a maximum inhibition value less than 30%. Hydrolysis of hazelnut proteins with pancreatin for 60 min also reduced the IgE-binding to less than 30% maximum inhibition (Wigotzki et al. 2000 a).

The vast majority of allergens able to sensitize *via* the GI tract belong to both prolamin superfamily (comprising the prolamin storage proteins of cereals, *ns*LTPs, 2S albumins and the  $\alpha$ -amylase inhibitors) and *cupin* superfamily (comprising the 11S legumin-like and 7S vicilin-like seed storage globulins). This two family of proteins demonstrate to have two properties which enable them to survive digestion: structural stability and abundance, respectively. These properties help enough of the protein to survive in a sufficiently intact form. Whilst abundance is an important factor, it is probably secondary to protein stability. Globulins are less well digested even when the food matrix is processed to remove the activity of anti-nutritional factors such as trypsin inhibitors and lectins. These proteins are partially or fully insoluble between pH 3.5 and pH 6.5 and therefore the limited solubility in the stomach environment. More in deep several studies demonstrated that the acidic chain is more susceptible to proteolysis than the basic ones (Plumb et al., 1989; Orunno and Morgan 2011). As well as the *ns*-LTP and the 2S albumin has been demonstrated extensively resistance to proteolysis by pepsin (Ciardiello et al., 2010; Asero et al., 2000).

These results suggest that the resistance to the SGF is not a discriminative factor for food allergens definition. Several studies demonstrated that to trigger initial sensitization the peptide fragments resulting from gastrointestinal proteolysis must be in a range of molecular weight between 3000 and 5000 Da (more than 25 residues) (Lehrer et al., 1996). Anyway, very small portions of material may escape lumen hydrolysis and are thus available for exposure to the gastrointestinal associated lymphoid tissues. Few data support the hypothesis that a protein need to be in an intact form to sensitize and, moreover, it is not clear whether there is a minimal size requirement for a peptide to be able to elicit allergic reaction (Fu 2002). Huby et al., 2000 suggested that the minimal size for a peptide to be recognized immunoglobulins on the surface of mast cells was at least 3 kDa.

The digestibility of a protein is greatly influenced by the *in vitro* assay conditions used. The pH variations and the concentration of enzyme could affect the estimation of allergenicity. Several predictive models of *in vitro* gastrointestinal digestion of proteins have been described in literature. Commonly allergens undergo a multistep static model that involves the sequential use of: pepsin, pancreatic proteases and extracted of human intestinal brush border membranes, in simulated gastric duodenal and jejunal environments, respectively. Methods to monitor the evaluation of protein allergenicity during artificial digestion procedures are based on HPLC and SDS-



PAGE/immunoblot analysis of intact and/or residual antigenic fragments (Figure 7). The *in vitro* digestion can be couple with model system for intestinal epithelial permeability to demonstrate the ability of peptides to surviving GI digestion and traslocate the intestinal barrier.

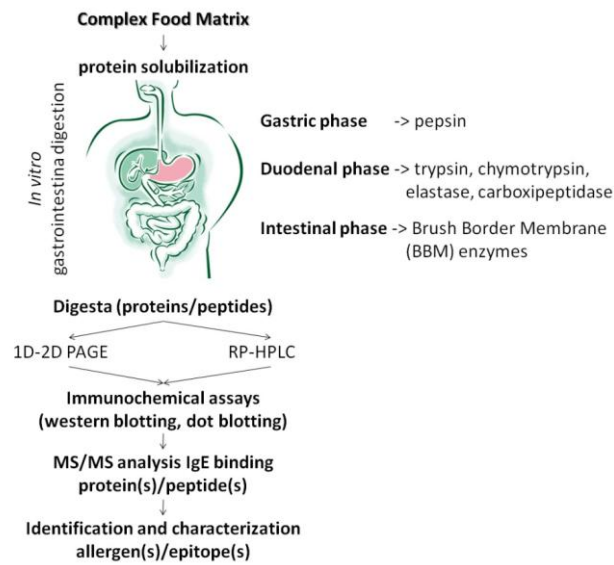


Figure 7. Flow chart of a static *in vitro* digestion model. The kinetic of digestion can be monitored at protein level by electrophoresis analysis whilst at peptide level by liquid chromatography. Total peptide/protein digest from digestion can be assayed for immunoreactivity by dot-blot analysis. RP-HPLC isolation of peptides followed by mass spectrometry analysis of immunoreactive peptides allowed the identification of epitope(s).

## 1.4 References

- Alisi C, Afferni C, Iacovacci P, Barletta B, Tinghino R, Butteroni C, Puggioni EM, Wilson IB, Federico R, Schininà ME, Ariano R, Di Felice G, Pini C (2001) Rapid isolation, characterization, and glycan analysis of Cup a 1, the major allergen of Arizona cypress (*Cupressus arizonica*) pollen. *Allergy* 56:978–984.
- Altmann F (2007) The role of protein glycosylation in allergy. *Int Arch Allergy Immunol* 142(2):99–115.
- Anderson JA, Song DD Adverse reactions to foods. American Academy of Allergy and Immunology Committee on Adverse reactions to Food/NIAID 1984. NIH publication 84-2442, Washington, pp. 1–6.
- Andersson K, Ballmer-Weber BK, Cistero-Bahima A, Ostling J, Lauer I, Vieths S, Lidholm J (2007) Enhancement of hazelnut extract for IgE testing by recombinant allergen spiking. *Allergy* 62: 897–904.
- Ansari P, Stoppacher N, Baumgartner S (2012) Marker peptide selection for the determination of hazelnut by LC-MS/MS and occurrence in other nuts. *Anal Bioanal Chem* 402(8):2607–15.
- Apweiler R, Hermjakob H, Sharon N (1999) On the frequency of protein glycosylation, as deduced from analysis of the SWISS-PROT database. *Biochim Biophys Acta* 1473(1):4–8.
- Asero R, Mistrello G, Roncarolo D, de Vries SC, Gautier MF, Ciurana CL, Verbeek E, Mohammadi T, Knul-Brettlova V, Akkerdaas JH, Bulder I, Aalberse RC, van Ree R (2000) Lipid transfer protein: a pan-allergen in plant-derived foods that is highly resistant to pepsin digestion. *Int Arch Allergy Immunol* 122(1):20–32.
- Astwood JD, Leach JN, Fuchs RL (1996) Stability of food allergens to digestion *in vitro*. *Nat Biotechnol* 14(10):1269–73.
- Akagawa M, Handoyo T, Ishii T, Kumazawa S, Morita N, Suyama K (2007) Proteomic analysis of wheat flour allergens. *J Agric Food Chem* 55:6863–6870.
- Akkerdaas JH, Wensing M, Knulst AC, Aalberse RC, Hefle SL, van Ree R (2003a) In vitro and in vivo characterization of hazelnut skin prick test extracts. *Arb Paul Ehrlich Inst Bundesamt Sera Impfstoffe Frankf A M.* 94:87–95.
- Akkerdaas JH, Wensing M, Knulst AC, Krebitz M, Breiteneder H, de Vries S, Penninks AH, Aalberse RC, Hefle SL, van Ree R (2003b) How accurate and safe is the diagnosis of hazelnut allergy by means of commercial skin prick test reagents? *Int Arch Allergy Immunol* 132:132–140.
- Akkerdaas JH, Schocker F, Vieths S, Versteeg S, Zuidmeer L, Hefle SL, Aalberse RC, Richter K, Ferreira F, van Ree R (2006) Cloning of oleosin, a putative new hazelnut allergen, using a hazelnut cDNA library. *Mol Nutr Food Res* 50:18–23.
- Barre A, Borges JP, Culerrier R, Rougé P (2005) Homology modelling of the major peanut allergen Ara h 2 and surface mapping of IgE-binding epitopes. *Immunol Lett* 100(2):153–8.
- Ben Rejeb S, Abbott M, Davies D, Querry J, Clérout C, Streng C, Delahaut P, Yeung JM (2003) Immunochemical-based method for detection of hazelnut proteins in processed foods. *J AOAC Int* 86(3):557–63.
- Beranová-Giorgianni S (2003) Proteome analysis by two-dimensional gel electrophoresis and mass spectrometry: strengths and limitations. *TrAC Trends in Analytical Chemistry* 22 (5):273–281.
- Beyer K, Grishina G, Bardina L, Grishin A, Sampson HA (2002) Identification of an 11S globulin as a major hazelnut food allergen in hazelnut-induced systemic reactions. *J Allergy Clin Immunol* 110(3):517–23.
- Bignardi C, Elviri L, Penna A, Careri M, Mangia A (2010) Particle-packed column versus silica-based monolithic column for liquid chromatography-electrospray-linear ion trap-tandem mass spectrometry multiallergen trace analysis in foods. *J Chromatogr A* 1217(48):7579–85.
- Boersema PJ, Mohammed S, Heck AJR (2008) Hydrophilic interaction liquid chromatography (HILIC) in proteomics. *Anal Bioanal Chem* 391(1): 151–159.

- Boye JI, Godefroy SB, Pulido OM. Immune - mediated adverse reactions to dietary proteins – in Allergen management in the food industry, edited Joyce I. Boye, John Wiley & Sons, Inc. 2010 pag 3.
- Bruijnzeel-Koomen C, Ortolani C, Aas K, Bindslev-Jensen C, Björkstén B, Moneret-Vautrin D, Wüthrich B (1995) Adverse reactions to food. European Academy of Allergology and Clinical Immunology Subcommittee. *Allergy* 50(8):623-35.
- Bublin M, Radauer C, Wilson IB, Kraft D, Scheiner O, Breiteneder H, Hoffmann-Sommergruber K (2003) Cross-reactive N-glycans of Api g 5, a high molecular weight glycoprotein allergen from celery, are required for immunoglobulin E binding and activation of effector cells from allergic patients. *FASEB J* 17:1697–1699.
- Cargile BJ, Stephenson JL (2004) An alternative to tandem mass spectrometry: isoelectric point and accurate mass for the identification of peptides. *Anal Chem* 76:267-275.
- Chehade M, Mayer L (2005) Oral tolerance and its relation to food hypersensitivities. *J Allergy Clin Immunol* 115:3-12.
- Ciardiello MA, Palazzo P, Bernardi ML, Carratore V, Giangrieco I, Longo V, Melis M, Tamburrini M, Zennaro D, Mari A, Colombo P (2010) Biochemical, immunological and clinical characterization of a cross-reactive nonspecific lipid transfer protein 1 from mulberry. *Allergy* 65(5):597-605.
- Colgrave ML, Goswami H, Howitt CA, Tanner GJ (2012) What is in a Beer? Proteomic Characterization and Relative Quantification of Hordein (Gluten) in Beer. *J Proteome Res* 11(1):386–396.
- Crespo JF, James JM, Fernandez-Rodriguez C, Rodriguez J (2006) Food allergy: nuts and tree nuts. *Br J Nutr Suppl* 2:S95-102.
- Cucu T, De Meulenaer B, Devreese B (2012) MALDI-based identification of stable hazelnut protein derived tryptic marker peptides. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess* 29(12):1821-31.
- De Knop KJ, Verweij MM, Grimmelikhuijsen M, Philipse E, Hagendorens MM, Bridts CH, De Clerck LS, Stevens WJ, Ebo DG (2011) Age-related sensitization profiles for hazelnut (*Corylus avellana*) in a birch-endemic region. *Pediatr Allergy Immunol* 22: 139–149.
- Ebner C, Hirschwehr R, Bauer L, Breiteneder H, Valenta R, Ebner H, Kraft D, Scheiner O (1995) Identification of allergens in fruits and vegetables: IgE cross-reactivities with the important birch pollen allergens Bet v 1 and Bet 2 (birch profilin). *J Allergy Clin Immunol* 95:962–969.
- Ehlert A, Demmel A, Hupfer C, Busch U, Engel KH (2009) Simultaneous detection of DNA from 10 food allergens by ligation-dependent probe amplification. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess* 26(4):409-18.
- European Parliament and Council, 2007. Directive 2007/68/EC of 27 November 2007 amending Directive 2000/13/EC as regards certain food ingredients. Official Journal of the European Communities L310/11.
- Fernández-Rivas M, Miles S. Food Allergies: Clinical and Psychosocial Perspectives. In Plant Food Allergens, ed. by E.N. Clare Mills, Peter R. Shewry, by Blackwell Science Ltd 2004 vol 1 pp. 1-23.
- Foetisch K, Westphal S, Lauer I, Retzek M, Altmann F, Kolarich D, Scheurer S, Vieths S (2003) Biological activity of IgE specific for cross-reactive carbohydrate-determinants. *J Allergy Clin Immunol* 111: 889–896.
- Fu TJ (2002) Digestion stability as a criterion for protein allergenicity assessment. *Ann N Y Acad Sci* 964:99-110.
- Garcia-Casado G, Sanchez-Monge R, Chrispeels MJ, Armentia A, Salcedo G, Gomez L (1996) Role of complex asparagine-linked glycans in the allergenicity of plant glycoproteins. *Glycobiology* 6(4): 471-477.
- Garino C, Zuidmeer L, Marsh J, Lovegrove A, Morati M, Versteeg S, Schilte P, Shewry P, Arlorio M, van Ree R (2010) Isolation, cloning and characterization of the 2S albumin, a new allergen from hazelnut. *Mol Nutr Food Res* 54:1257-1265.
- Gern JE, Yang E, Evrard HM, Sampson HA (1991) Allergic reactions to milk-contaminated "nondairy" products. *N Engl J Med* 324(14):976–979.

- Gygi SP, Rist B, Griffin TJ, Eng J, Aebersold R (2002) Proteome analysis of low-abundance proteins using multidimensional chromatography and isotope-coded affinity tags. *J Proteome Res.* 1(1):47-54.
- Haas-Lauterbach S, Immer U, Richter M, Koehler P (2012) Gluten fragment detection with a competitive ELISA. *J AOAC Int.* 95(2):377-81.
- Hansen KS, Ballmer-Weber BK, Lüttkopf D, Skov PS, Wüthrich B, Bindslev-Jensen C, Vieths S, Poulsen LK. (2003) Roasted hazelnuts – allergenic activity evaluated by double-blind, placebo-controlled food challenge. *Allergy* 58(2):132-8.
- Han X, Jin M, Breuker K, McLafferty FW (2006) Extending top-down mass spectrometry to proteins with masses great than 200 kilodaltons. *Science* 314:109–112.
- Haselberg R, de Jong GJ, Somsen GW (2007) Capillary electrophoresis-mass spectrometry for the analysis of intact proteins. *J Chromatogr A* 1159:81.
- Herrero M, Simó C, García-Cañas V, Ibáñez E, Cifuentes A (2012) Foodomics: MS-based strategies in modern food science and nutrition. *Mass Spectrom Rev* 31(1):49-69.
- Houston NL, Lee DG, Stevenson SE, Ladics GS, Bannon GA, McClain S, Privalle L, Stagg N, Herouet-Guicheney C, MacIntosh SC, Thelen JJ (2011) Quantitation of soybean allergens using tandem mass spectrometry. *J Proteome Res.* 10(2):763-73.
- Huby JDR, Dearman RJ, Kimber I (2000) Why Are Some Proteins Allergens? *Toxicol Sci* 55:235-246.
- Jégou S, Douliez JP, Molle D, Boivin P, Marion D (2000) Purification and structural characterization of LTP1 polypeptides from beer. *J Agric Food Chem* 48:5023–5029.
- Jégou S, Douliez JP, Molle D, Boivin P, Marion D (2001) Evidence of the glycation and denaturation of LTP1 during the malting and brewing process. *J Agric Food Chem* 49:4942–4949.
- Johnson RS, Davis MT, Taylor JA, Patterson SD (2005) Informatics for protein identification by mass spectrometry. *Methods* 35(3):223-36.
- Keeler M, Letarte J, Hattrup E, Hickman F, Haynes PA (2007) Two-Dimensional Differential In-Gel Electrophoresis (DIGE) of Leaf and Roots of *Lycopersicon esculentum*. *Methods in Molecular Biology*. Walker JM 355:157-174.
- Kim J, Chun MS, Choi K, Chung DS (2009) Large volume stacking using an EOF pump in NACE-MS. *Electrophoresis* 30(6):1046-51.
- Klein E, Baudner S, Guenther HO (1985) Immunochemical determination of hazelnut proteins by the electroimmunodiffusion method of Laurell. I. Optimized methodology. *Z Lebensm –Unters Forsch* 180(1): 30-35, Aachen FGR.
- Knippels LM, Penninks AH (2003) Assessment of the allergic potential of food protein extracts and proteins on oral application using the brown Norway rat model. *Environ Health Perspect* 111(2):233-8.
- Koeppel E, Stadler M, Luethy J, Huebner P (1998) Detection of wheat contamination in oats by polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA) *Zeitschrift fuer Lebensmittel-Untersuchung und -Forschung A* 206(6):399-403.
- Koppelman SJ, Knulst AC, Koers WJ, Penninks AH, Peppelman H, Vlooswijk R, Pigmans I, van Duijn G, Hessing, M (1999) Comparison of different immunochemical methods for the detection and quantification of hazelnut proteins in food products. *J Immunol M* 229 (1-2):107-120.
- Kvasnicka F (2003) Proteomics: general strategies and application to nutritionally relevant proteins. *J Chromatogr B Analyt Technol Biomed Life Sci* 787(1):77-89.

- Lauer I, Foetisch K, Kolarich D, Ballmer-Weber BK, Conti A, Altmann F, Vieths S, Scheurer S (2004) Hazelnut (*Corylus avellana*) vicilin Cor a 11: molecular characterization of a glycoprotein and its allergenic activity. *Biochem J*. 383(Pt 2):327-34.
- Lehrer SB and O'Neil CE (1992) Occupational reactions in the food industry. *Food Technol* 46:153-156.
- Lehrer SB, Horner WE, Reese G (1996) Why are some proteins allergenic? Implications for biotechnology. *Crit Rev Food Sci Nutr* 36(6):553-64.
- Lemke PJ, Taylor SL Allergic reactions and food intolerances, in "Nutritional Toxicology," Ed. Kotsonis FN, Mackey M, Hjelle J, pp. 117-137.
- Lu B, Xu T, Park SK, Yates JR (2009) Shotgun protein identification and quantification by mass spectrometry. *Methods Mol Biol* 564:261-288.
- Lüttkopf D, Müller U, Skov PS, Ballmer-Weber BK, Wüthrich B, Skamstrup Hansen K, Poulsen LK, Kästner M, Hausteint D, Vieths S (2002) Comparison of four variants of a major allergen in hazelnut (*Corylus avellana*) Cor a 1.04 with the major hazel pollen allergen Cor a 1.01. *Mol Immunol* 38(7):515-25.
- Mamone G, Picariello G, Addeo F, Ferranti P (2011) Proteomic analysis in allergy and intolerance to wheat products. *Expert Rev Proteomics* 8(1):95-115.
- Mann M, Hendrickson RC, Pandey A (2001) Analysis of proteins and proteomes by mass spectrometry. *Annu Rev Biochem* 70:437-73.
- Monaci L, Tregot V, van Hengel AJ, Anklam E (2006) Milk allergens, their characteristics and their detection in food: a review. *Eur Food Res Technol* 223:149–179.
- Monaci L, Visconti A (2009) Mass spectrometry-based proteomics methods for analysis of food allergens. *TRAC-Trends Anal Chem* 28(5): 581-591.
- Monaci L, Losito I, Palmisano F, Visconti A (2010) Identification of allergenic milk proteins markers in fined white wines by capillary liquid chromatography-electrospray ionization-tandem mass spectrometry. *J Chromatogr A* 1217(26):4300-5.
- Monaci L, Losito I, Palmisano F, Godula M, Visconti A (2011 a) Towards the quantification of residual milk allergens in caseinate-fined white wines using HPLC coupled with single-stage Orbitrap mass spectrometry. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess* 28(10):1304-14.
- Monaci L, Losito I, Palmisano F, Visconti A (2011 b) Reliable detection of milk allergens in food using a high-resolution, stand-alone mass spectrometer. *J AOAC Int* 94(4):1034-42.
- Mills ENC, Jenkins JA, Bannon GA. Plant Seed Globulin Allergens Plant. In *Plant Food Allergens*, Ed by E.N. Clare Mills, Peter R. Shewry, by Blackwell Publishing Ltd 2004 vol 2 pp 24.
- Moreno JF, and Clemente A (2008) 2S Albumin Storage Proteins: What Makes them Food Allergens? *Open Biochem J* 2: 16–28.
- Müller U, Lüttkopf D, Hoffmann A, Petersen A, Becker WM, Schocker F, Niggemann B, Altmann F, Kolarich D, Hausteint D, Vieths S (2000) Allergens in raw and roasted hazelnuts (*Corylus avellana*) and their cross-reactivity to pollen. *European Food Research and Technology*. 212(1): 2-12.
- Natarajan SS, Krishnan HB, Lakshman S, Garrett WM (2009) An efficient extraction method to enhance analysis of low abundant proteins from soybean seed. *Anal Biochem* 394(2):259-68.
- Nishiyama T, Kimura N, Jitsuhara Y, Uchida M, Ochi F, Yamaguchi H. (2000) The N-Glycans Protect Proteins from Protease Digestion through Their Binding Affinities for Aromatic Amino Acid Residues. *Biochem*. 127:427-433.
- Opiteck GJ, Jorgenson JW *Comprehensive* (1997) On-Line LC/LC/MS of Proteins. *Anal Chem* 69:1518–1524.

- Orruño E, Morgan MRA (2011) Resistance of purified seed storage proteins from sesame (*Sesamum indicum* L.) to proteolytic digestive enzymes. *Food Chemistry* 128:923–929.
- Pastorello EA, Vieths S, Pravettoni V, Farioli L, Trambaioli C, Fortunato D, Lüttkopf D, Calamari M, Ansaloni R, Scibilia J, Ballmer-Weber BK, Poulsen LK, Wütrich B, Hansen KS, Robino AM, Ortolani C, Conti A (2002) Identification of hazelnut major allergens in sensitive patients with positive double-blind, placebo-controlled food challenge results. *J Allergy Clin Immunol* 109(3):563-70.
- Picariello G, Mamone G, Addeo F, Ferranti P (2011) The frontiers of mass spectrometry-based techniques in food allergenomics. *J Chromatogr A* 1218(42):7386-98.
- Picariello G, Mamone G, Nitride C, Addeo F, Camarca A, Vocca I, Gianfrani C, Ferranti P (2012) Shotgun proteome analysis of beer and the immunogenic potential of beer polypeptides. *J Proteomics* 75(18):5872-82.
- Picariello G, Mamone G, Addeo F, Nitride C, Ferranti P. Proteomic-based techniques for the characterization of food allergens. in *Foodomics: Advanced Mass Spectrometry in Modern Food Science and Nutrition*, First Edition. Ed. by Alejandro Cifuentes 2013 John Wiley & Sons, Inc Chapter3, pp. 69-99.
- Platteau C, De Loose M, De Meulenaer B, Taverniers I (2011) Quantitative Detection of Hazelnut (*Corylus avellana*) in Cookies: ELISA versus Real-Time PCR. *J Agric Food Chem* 59:11395–11402.
- Plumb GW, Carr HJ, Newby VK, Lambert N (1989) A study of the trypsinolysis of pea 11S globulin. *Biochim Biophys Acta* 999:281–288.
- Rapid Alert System for Food and Feed (RASFF) 2011, [http://ec.europa.eu/food/food/rapidalert/index\\_en.htm](http://ec.europa.eu/food/food/rapidalert/index_en.htm)
- Robotham JM, Hoffman GG, Teuber SS, Beyer K, Sampson HA, Sathe SK, Roux KH (2009) Linear IgE-epitope mapping and comparative structural homology modeling of hazelnut and English walnut 11S globulins. *Molecular Immunology* 46:2975–2984.
- Sampson HA (1999) Food allergy. Part 1: Immunopathogenesis and clinical disorders. *J Allergy Clin Immunol* 103(5):717-728.
- Schnell S, Herman RA (2009) Should digestion assays be used to estimate persistence of potential allergens in tests for safety of novel food proteins? *Clin Mol Allergy* 7:1.
- Schocker F, Lüttkopf D, Scheurer S, Petersen A, Cisteró-Bahima A, Enrique E, San Miguel-Moncín M, Akkerdaas J, van Ree R, Vieths S, Becker WM (2004) Recombinant lipid transfer protein Cor a 8 from hazelnut: a new tool for in vitro diagnosis of potentially severe hazelnut allergy. *J Allergy Clin Immunol* 113(1):141-7.
- Shefcheck KJ, Callahan JH, Musser SM (2006) Confirmation of peanut protein using peptide markers in dark chocolate using liquid chromatography tandem mass spectrometry (LC-MS/MS). *J Agric Food Chem* 54:7953-7959.
- Sicherer SH, Burks AW, Sampson HA (1998) Clinical Features of Acute Allergic Reactions to Peanut and TreeNuts in Children. *Pediatrics* 102(1).
- Skamstrup Hansen K, Bindslev-Jensen C, Stahl Skov P, Sparholt SH, Nordskov Hansen G, Niemeijer NR, Malling HJ, Poulsen LK (2001) Standardization of food allergen extracts for skin prick test *Journal of Chromatography B*, 756:57–69.
- Taylor SL, Lemanske RF Jr, Bush RK, Busse WW (1987) Food allergens: structure and immunologic properties. *Ann Allergy* 59(5 Pt 2):93-9.
- Taylor SL (1996) Genetically engineered foods: commercial potential safety and allergenicity. *Food Australia* 48:308-311.
- Taylor SL, Hefle S L (2001) Food allergies and other food sensitivities. *Food technology* 55:9.
- Tretter V, Altmann F, Kubelka V, Marz L, Becker WM (1993) Fucose  $\alpha(1,3)$ -linked to the core region of glycoprotein N-glycans creates an important epitope for IgE from honeybee venom allergic individuals. *Int Arch Allergy Immunol* 102: 259–266.

- van Ree R, Cabanes-Macheteau M, Akkerdaas J, Milazzo JP, Loutelier-Bourhis C, Rayon C, Villalba M, Koppelman S, Aalberse R, Rodriguez R, Faye L, Lerouge P (2000)  $\beta(1,2)$ -Xylose and  $\alpha(1,3)$ - Fucose Residues Have a Strong Contribution in IgE Binding to Plant Glycoallergens. *J Biol Chem* 275(15):11451-11458.
- Verweij MM, Hagendorens MM, De Knop KJ, Bridts CH, De Clerck LS, Stevens WJ, Ebo DG (2011) Young infants with atopic dermatitis can display sensitization to Cor a 9, an 11S legumin-like seed-storage protein from hazelnut (*Corylus avellana*). *Ebo Pediatr Allergy Immunol* 22(2):196-201.
- Verweij MM, Hagendorens MM, Trashin S, Cucu T, De Meulenaer B, Devreese B, Bridts CH, De Clerck LS, Ebo DG (2012) Age-Dependent Sensitization to the 7S-Vicilin-Like Protein Cor a 11 From Hazelnut (*Corylus avellana*) in a Birch- Endemic Region. *J Investig Allergol Clin Immunol* 22(4): 245-251.
- Vieths S, Zagon J, Fischer K, Dehne LI, Bögl KW (1995) Verarbeitungsbedingte Einflüsse auf das allergene Potential von Lebensmitteln. Die Sojaallergie als Modellbeispiel *Lebensmittelchemie* 49:29-31 (in German)
- Vieths S, Reind J, Müller U, Hoffmann A, Haustein D (1999) Digestibility of peanut and hazelnut allergens investigated by a simple in vitro procedure. *European Food Research and Technology* 209(6):379-388.
- Vieths S, Scheurer S, Ballmer-Weber B (2002) Current Understanding of Cross-Reactivity of Food Allergens and Pollen. *Ann N Y Acad Sci* 964:47-68.
- Weber D, Raymond P, Ben-Rejeb S, Lau B (2006) Development of a liquid chromatography-tandem mass spectrometry method using capillary liquid chromatography and *nano*-electrospray ionization-quadrupole time-of-flight hybrid mass spectrometer for the detection of milk allergens. *J Agric Food Chem* 54(5):1604-10.
- Wigotzki M, Steinhart H, Paschke A (2001) Determination of the allergenicity of various hazelnut products by immunoblotting and enzyme allergosorbent test inhibition *J Chromatogr B* 756:239-48.
- Wijesinha-Bettoni R, Alexeev Y, Johnson P, Marsh J, Sancho AI, Abdullah SU, Mackie AR, Shewry PR, Smith LJ, Mills EN (2010) The Structural Characteristics of Nonspecific Lipid Transfer Proteins Explain: Their Resistance to Gastrointestinal Proteolysis. *Biochemistry* 49:2130-2139.
- Worm M, Hompes S, Fiedler EM, Illner AK, Zuberbier T, Vieths S (2009) Impact of native, heat-processed and encapsulated hazelnuts on the allergic response in hazelnut-allergic patients. *Clin Exp Allergy* 39(1):159-66.
- Yagami T, Haishima Y, Nakamura A, Osuna H, Ikezawa Z (2000) Digestibility of allergens extracted from natural rubber latex and vegetable foods. *J Allergy Clin Immunol* 106(4):752-62.
- Yates JR 3rd (2004) Mass spectral analysis in proteomics *Annu Rev Biophys Biomol Struct* 33:297-316.
- Zuidmeer L, Goldhahn K, Rona RJ, Gislason D, Madsen C, Summers C, Sodergren E, Dahlstrom J, Lindner T, Sigurdardottir ST, McBride D, Keil T (2008) The prevalence of plant food allergies: a systematic review. *J Allergy Clin Immunol* 121(5):1210-1218.

**2.**

**Section 1: Proteomic characterization of hazelnut allergens.**



## 2.1 Aim of the study

Plant proteins can be classified into families and super-families on the basis of their sequence relationships. Database interrogation demonstrated that only a small portion of these families contain identified and characterized allergens. The missing of genomic and proteomic sequences challenges the identification of allergens through the classical proteomic approach. The overall aim of this thesis was to address advanced proteomics approaches to overcome limits of the classical approach and obtain a complete description of allergens of plant origin include legumes, nuts, and seeds, using as model the hazelnut allergens.

## 2.2 Optimization of proteomic sample preparation procedures for comprehensive protein characterization of hazelnuts system.

### 2.2.1 Material

Three hazelnut cultivars from Regione Campania (Mortarella, San Giovanni, Tonda) and one from Oregon were utilized in this study. Trifluoroacetic acid (TFA), dithiothreitol (DTT), iodoacetamide, phenylmethanesulfonylfluoride (PMSF),  $\alpha$ -cyano-4-hydroxycinnamic acid (4-CHCA), sinapinic acid and phosphate buffer (PBS) were provided by Sigma-Aldrich (Milan, Italy). The electrophoresis reagents were from GE-healthcare (Milan, Italy). Ammonium bicarbonate (AMBIC), reagents and HPLC-grade solvents were from Carlo Erba (Milan, Italy) and were used without any further purification. Sequencing-grade modified trypsin was supplied by Promega (Madison, WI, USA). The skin prick test (SPT) solution was from Lofarma (Milan, Italy).

### 2.2.2 Methods

#### 2.2.2.1 Protein Extraction

Shelled raw hazelnuts were ground using an electric grinder to a fine powder, and defatted by stirring twice for 1 h in five volumes (w/v) of diethyl ether. Initially different solubilization buffers and SDS staining techniques were tested in order find the optimal condition of analysis for hazelnuts proteins. The 1D and 2D maps of proteins extracted with denaturing and reducing agents (7M urea, 2M thiourea, 2% CHAPS, 0.5M DTT) and with phosphate/saline buffered (0.1M PBS, pH 7.5, 1/10, w/v) were compared. Gels were stained using colloidal Coomassie G250 (Invitrogen) and fluorescent chromophore-staining (SYPRO Ruby) that is much more sensitive. Since some of the seed storage proteins undergo a post-translational modifications that cleavage in at least two subunits linked *via* disulphide bridges, the phosphate buffer was preferred as solubilizing agents to preserving the native protein structure. Proteins were extracted into 0.1M PBS, (pH 7.5; 1/10, w/v) containing protease inhibitor (1 mM PMSF) by stirring overnight at 4 °C. After centrifugation (10000 g, 30 min, 4 °C), the supernatant was collected and filtered through a 0.2 mm cellulose acetate filter (Millipore, Darmstadt, Germany). The protein concentration was determined by the Bradford assay. For 2 DE and mass spectrometry analyses, the hazelnut proteins were reduced with 10 mM DTT in 0.3 M Tris–HCl containing 6 M guanidine/HCl at pH 8.0, for 60 min at 56 °C and alkylated with 55 mM iodoacetamide, dissolved in the same buffer above, for 30 min at room temperature in the dark. Low molecular weight compounds were removed from the sample by filtration through a PD-10 desalting column (GE Healthcare) using for elution a solution of 5% acetic acid. Fractions that shown absorbance at 280 nm, were pooled together and lyophilized.

#### 2.2.2.2 SDS-PAGE analysis

The hazelnut extracts and were separated by 1D-SDS-PAGE electrophoresis using 12% polyacrylamide gels. Proteins (12 µg for well) were resuspended in 20 µL of the Laemli buffer (0.125 M Tris–HCl pH 6.8, 5% SDS, 20% Glycerol, 5% (w/v)  $\beta$ -mercaptoethanol, 0.02% bromophenol blue) and boiled for 5 min. Electrophoresis was also performed in non-reducing conditions, where  $\beta$ -mercaptoethanol was omitted in the solubilization buffer. Electrophoresis was carried out using the Tris–glycine–SDS buffer system (25 mM Tris, 192 mM glycine and 0.1% SDS) on a Mini-PROTEAN® Tetra Handcast Systems.

#### 2.2.2.3 Two-dimensional IEF/SDS-PAGE

Hazelnut extracts (200 µg) were separated by 2DE, as described by O'Farrell et al., (1975). The 1D step was performed using an Ettan IPGphor II system (GE healthcare). Immobilized pH 3-10 L gradient strips (11 cm) were rehydrated overnight with 200 µg of reduced and alkylated crude hazelnut protein extracts previously suspended in 200 µL of IPG strip rehydration buffer (8 M Urea; 2% CHAPS; 20 mM DDT; 2% IPG buffer; 0.002% bromophenol blue). The proteins were focused up to 11000 Vh at a maximum voltage of 6000 V at 20 °C. IPG strips were soaked in the equilibration solution (6 M Urea, 2% w/v SDS, 30% w/v glycerol, 50 mM Tris-HCl, pH 8.8, 0.002% bromophenol blue) for 15 min with 1% DTT (w/v) and 15 min with 2.5% iodoacetamide (w/v) at room temperature. The 2D-SDS-PAGE was carried out using a HOEFER SE 600 series system (Amersham), separating proteins on a

12% hand-cast SDS-polyacrylamide gel (16 x 14 cm) using the Tris-glycine-SDS buffer system at 15 mA/gel for 30 min and then at 30 mA/gel. The protein spots were stained with Coomassie Brilliant Blue (CBB) R-250. The 2-DE protein patterns were recorded as digitalized images using an ImageScanner (Amersham Biosciences) operated by the software Lab-Scan 3.00 (Amersham Biosciences). Spot analyses were performed using the ImageMaster 2D Platinum software 6.0 (Amersham Biosciences). The spots were excised and the proteins were submitted to mass spectrometry analysis.

#### 2.2.2.4 Protein in-gel digestion

The protein spots were destained by repeated washing with 25 mM AMBIC/acetonitrile (1/1, v/v). The proteins were reduced for 1 h at 57 °C with 10 mM DTT in 25 mM AMBIC and alkylated for 30 min at room temperature with 55 mM iodoacetamide in 25 mM AMBIC. The digestion was carried out overnight at 37 °C with modified proteomic grade trypsin (12.5 ng/μl) in 25 mM AMBIC. The peptides were extracted three times in 5% formic acid/acetonitrile (1/1, v/v) and finally dried in a “speed-vac” centrifuge. Prior to MS analysis, peptide digests were desalted using C<sub>18</sub> Zip-Tip pre-packed micro-columns (Millipore, Bedford, MA, USA), previously equilibrated with 0.1% TFA and eluted with 50% acetonitrile (v/v) containing 0.1% TFA (v/v).

#### 2.2.2.5 Gel filtration isolation of low molecular weight proteins

Proteins extracted in 20 mM MES, 150 mM NaCl pH 7 (1/10, w/v) were applied to a (1.6x60 cm) Superdex S75 preparative grade equilibrated with 50 mM Tris/HCl, 150 mM NaCl pH 7.56, at a flow rate of 1.0 mL/min. The absorbance was monitored at 220 and 280 nm. The column was pre-equilibrated with a set of gel filtration molecular weight standards (Bio-Rad, Hertfordshire, UK. Bovine thyroglobulin (Mr 670 kDa), bovine gamma globulin (Mr 158 kDa), chicken ovalbumin (Mr 44 kDa), horse myoglobin (Mr 17 kDa), and additionally with apoprotein (Mr 6.5 kDa) (Sigma Aldrich).

#### 2.2.2.6 RP-HPLC separation of hazelnut proteins

PBS soluble proteins were fractionated by Reversed Phase (RP)-HPLC using an HP 1100 Agilent Technology modular system (Palo Alto, CA, USA) equipped with a Vydac (Hesperia, CA, USA) C8 column (208TP52, 5μm, 2.1x250 mm). After 10 min of isocratic elution using 25% solvent B (0.1% TFA in acetonitrile, v/v), a fractionated step was applied: 25-30% B for 5 min, 30-35% B for 30 min, 35-50% B for 60 min, 50-55% B for 15 min at a flow rate of 0.200 mL/min. Solvent A was 0.1% TFA in water (v/v). For each analysis, approximately 200 μg of hazelnut proteins, dissolved in 0.1% TFA, were injected. Column effluents were monitored by UV detection at 220 and 280 nm. Protein fractions were manually collected and used for mass spectrometry analysis and electrophoresis separation either directly or after concentration in a speed-vac. Alternatively, the samples were lyophilized prior to enzymatic digestion.

#### 2.2.2.7 MALDI-TOF MS

Spectra were acquired using a Voyager DE Pro mass spectrometer (PerSeptive BioSystems, Framingham, MA, USA) equipped with a N<sub>2</sub> laser (λ= 337 nm). The instrument operated at an accelerating voltage of 20 or 25 kV. The mass spectra of the peptides were acquired in the reflector mode using 4-CHCA (10 mg/mL in 50% acetonitrile/0.1% TFA). The analysis of protein samples was carried out in the linear ion mode using sinapinic acid (10 mg/mL in 50% acetonitrile/0.1% TFA) as the matrix. Typically, 250 laser shots were accumulated for each spectrum. External mass calibration was performed with commercial standard peptide/protein mixtures (Sigma). The data were elaborated with the Data Explorer 4.0 software (PerSeptive BioSystems).

#### 2.2.2.8 Shotgun proteomic analysis by nano-HPLC-ESI MS/MS

Protein samples were hydrolyzed with sequencing grade modified trypsin (Promega, Madison, WI, USA) at an enzyme/substrate ratio of 1:100 w/w in 50 mM ammonium bicarbonate, pH 8.0, overnight at 37 °C. The samples were desalted using Sep-Pak® cartridges prior to nanoHPLC -MS/MS analysis. nano-flow LC-ESI MS/MS analysis was carried out using an Ultimate 3000 HPLC (Dionex, Sunnydale, CA, USA) coupled to a Q-STAR mass spectrometer (Applied BioSystems, Framingham, USA). The eluents were (A) 5% ACN in 0.1% FA and (B) 80% ACN in 0.08% FA. The peptides were loaded into a C<sub>18</sub> loading cartridge (LC Packings, USA) and separated with a C<sub>18</sub>PepMap100 column (15 cm length, 75 μm ID, 300 Å [LC Packings]), using a linear gradient of 5-40% B over 60 min at a constant flow rate of 300 nL/min. LC-MS/MS experiments were performed in the information-dependent acquisition (IDA) mode. Precursor ions were selected using the following MS to MS/MS switch criteria: ions greater than m/z 400, charge states 2 to 4, intensity exceeding 15 counts, former target ions were excluded for 30 s and ion tolerance was 50.0 mmu. CID was used to fragment multiple charged ions and nitrogen was used as the collision gas. The raw spectra files were used to generate text files in mascot generic file format (.mgf), which were submitted to the Mascot ver. 2.3 (<http://www.matrixscience.com>) and Batch-tag (Protein Prospector, University of California San Francisco, USA) search engines.

### 2.2.3 Results and discussion

#### 2.2.3.1 Electrophoretic Comparison of Seed Proteins Among Varieties by 1D and 2D-PAGE

The electrophoretic profile did not appreciably changed when the hazelnuts proteins were extracted with a denaturing/reducing buffer confirming that the prevalent allergens are saline-soluble proteins.

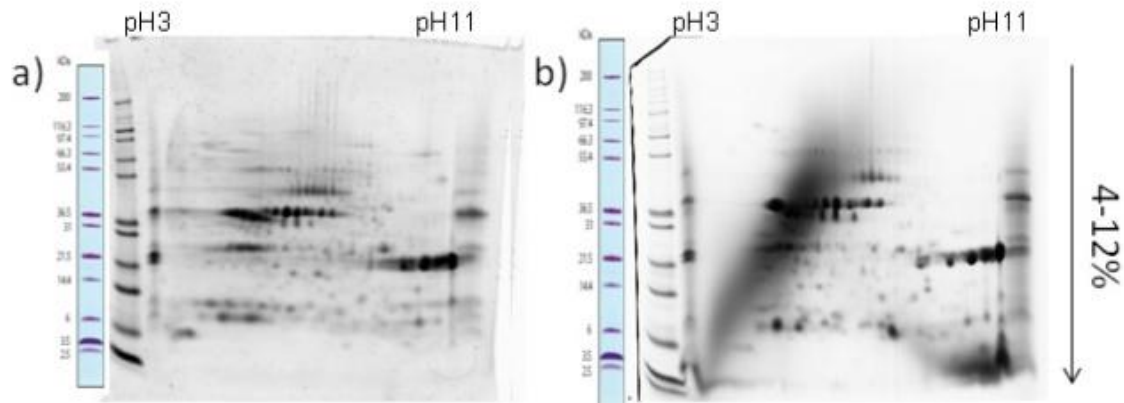


Figure 8. Comparison of different extraction buffer and gel staining solution. 2D IEF-SDS PAGE of proteins extracted with phosphate/saline buffered – sypro staining (panel a); 2D IEF-SDS PAGE of proteins extracted with Urea/Thiourea buffer – Comassie staining (panel b).

The lack of genomic and proteomic sequence of hazelnut challenges the complete characterization of allergens. The extreme high similarity among the 1D electrophoretic maps (Figure 9) and the profile obtained by the shotgun proteome technology of the protein extracted from the four hazelnut varieties suggests that there is no cultivar-dependent proteins expression, associated at least with the major storage proteins.

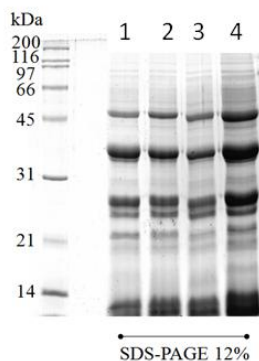


Figure 9. CBB R250 stained SDS-PAGE analysis of crude protein extracts from three hazelnut cultivars autochthonous of Regione Campania (lane 2: Mortarella, lane 3: San Giovanni, lane 4: Tonda) and one from Oregon, USA (lane 1).

The 2D map (Figure 8) shows the intrinsically high complicity of hazelnut storage protein's pattern in the presence of several spots with very similar molecular weight and pIs. This heterogeneity may suggest the presence of same or similar gene products. This characteristic occurs very frequently in seeds storage proteins because of their only function to provide nitrogen to the germ. This approach allowed to individuate only the most abundant hazelnut allergens on the 2D map, as for instance the 11S acid and basic chains (*Cor a 9*; UNIPROT entry: Q8W1C2, score 642); the 7S glycoprotein (*Cor a 11*; UNIPROT entry: Q8S4P9, score 293) with the typical glycoprotein pattern. Due to its intrinsic pitfalls, the classical 2D-based proteomic approach was unsuccessful to identify many of the minor spots and other protein components that probably have not been annotated in genomic and protein databases so far.

#### 2.2.3.2 Isolation and characterization of Low-Molecular Weight Proteins

The combination of 1D-2D electrophoresis with mass spectrometry analysis is a typical proteomic approach which allows very excellent performances in characterization of abundant proteins whose genomic and proteomic sequences are present in bioinformatics online tools. This performance decreases for low-abundant proteins. The predominance of higher molecular weight hazelnut storage proteins, for instance the 11S globulin like protein and the 7S vicilin seeds, hind the characterization of less abundant and lower molecular weight proteins (Natarajan SS et al., 2009) such as ns-lipid transfer proteins, profilins, and members of the Bet v 1-related family (Roux K.H et al., 2003). In order to overcome this lack and make possible a more complete characterization of hazelnut allergens, the protein extract was applied to a gel filtration column for the enrichment of the low molecular weight fraction.

Collected fractions were separated by 1D electrophoresis and characterized by peptide mass fingerprinting of the “*in gel*” tryptic digested bands. The gel filtration profile (Figure 10, panel a) showed the presence of a predominant sharp peak of about 160 kDa where were eluted the 11S globulin and the 7S vicilin and an unresolved complex mixture (hump) of low molecular weight proteins in a range between 17 and 6.5 kDa.

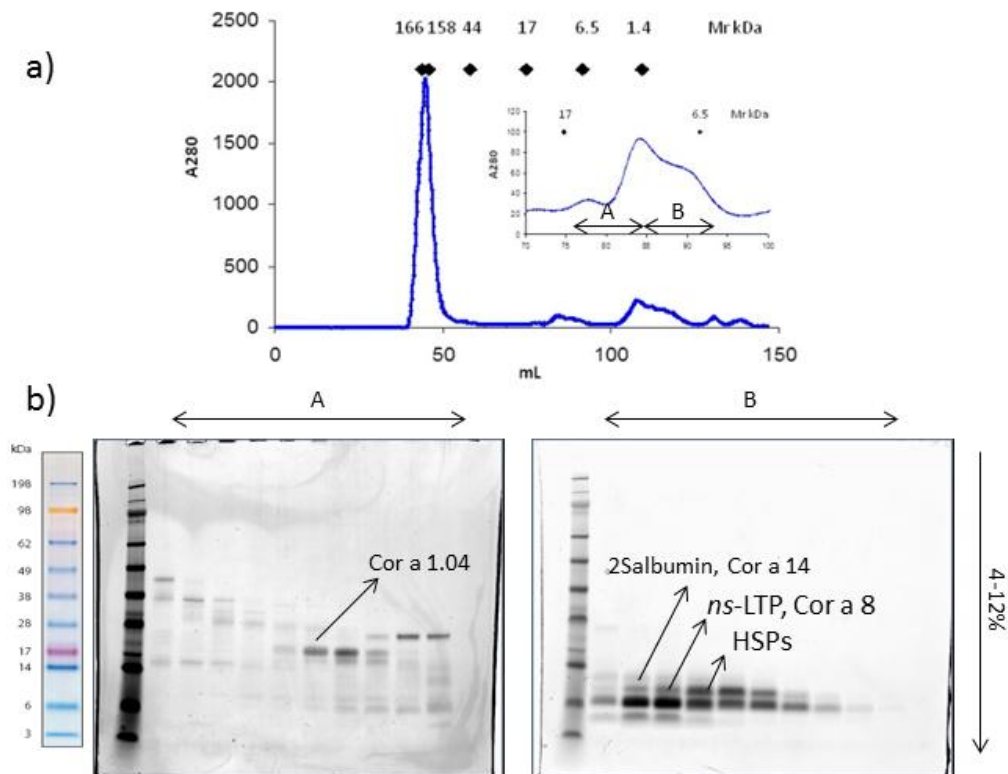


Figure 10. (panel a) Gel-filtration chromatographic fractionation of hazelnut proteins (in the inset an enlargement of low molecular weight fraction is reported). Molecular weight standards were bovine thyroglobulin (Mr 670 kDa), bovine gamma globulin (Mr 158 kDa), chicken ovalbumin (Mr 44 kDa) and horse myoglobin (Mr 17 kDa) indicated by filled diamonds; (panel b) CBB R250 stained 1D PAGE of collected fractions in the range of MW from 17 to 6.5 kDa.

The low molecular fraction was separated by 1D electrophoresis (Figure 10, panel b) and the proteins were identified by peptide mass fingerprinting of the *in gel* trypsin digested band. The chromatographic step allowed the identification of the *Cor a 1.0402* variant, homologues to *Bet v 1*, the major white birch pollen antigen (UNIPROT entry: Q9FPK4); the heat shock proteins (HSPs) (UNIPROT entry: Q9ZPQ0); the *ns*-Lipid Transfer Protein (*ns*-LTP) (*Cor a 8*; UNIPROT entry: Q9ATH2, score: 140, coverage: 57%); the 2S albumin (*Cor a 14*; UNIPROT entry: D0PWG2, score 223, coverage: 40.8%).

The only study two studies, reported in literature, aimed to the structural characterization of the 2S albumin are based on the cloned protein (Garino et al., 2010) and for this reason lacks of any information about posttranslational modifications. The 1D-SDS analysis of the gel filtration fractions containing the 2S albumin, analyzed in presence and absence of reducing agents (Figure 11) and the mass spectrometry analysis of the *in gel* tryptic digest, confirmed the post-translational proteolytic cleavage typically described for other storage proteins (Moreno et al., 2008) that split the protein in at least two subunits of about 10 and 6 kDa, linked via disulfide bridges.

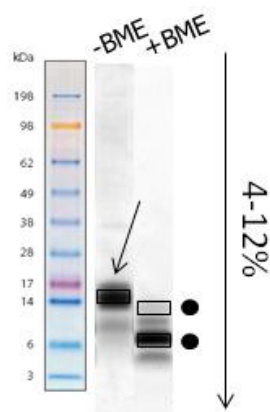


Figure 11. CBB R250 stained SDS-PAGE analysis of 2S albumin fractionated by gel filtration chromatography, treated in presence (-BME) and absence of  $\beta$ -mercaptoethanol as reducing agents (+BME). The intact form of 2S is labelled with the arrow whilst the two chains are labelled with filled circle.

### 2.2.3.3 RP-HPLC fractionation of PBS extracted proteins from raw hazelnut

The C8 RP-HPLC gradients parameters were optimized to provide high resolution separation of proteins. Crude hazelnut extracts, before and after treatment with reducing agents, was separated by (RP) HPLC-ESI-MS and eluted fractions were monitored by SDS-PAGE. Proteins identification was carried out by mass spectrometry analysis of the tryptic peptides derived from both the digestion of the collected fractions and the gel bands. The chromatogram (Figure 12, panel a) of native proteins showed the presence of two main peaks where the 11S globulin like protein (peaks 1 and 2 in figure 12) was eluted, followed by a series of smaller unresolved peaks (peaks 3-8 in figure 12) corresponding to the 7S vicilin which, because of the glycans, slither along the column. The 1D electrophoresis analysis of the fractions, carried out under non-reducing condition, showed the presence of minor proteins, eluted along all the chromatogram without resolution, which identification were hidden by the most abundant ones.

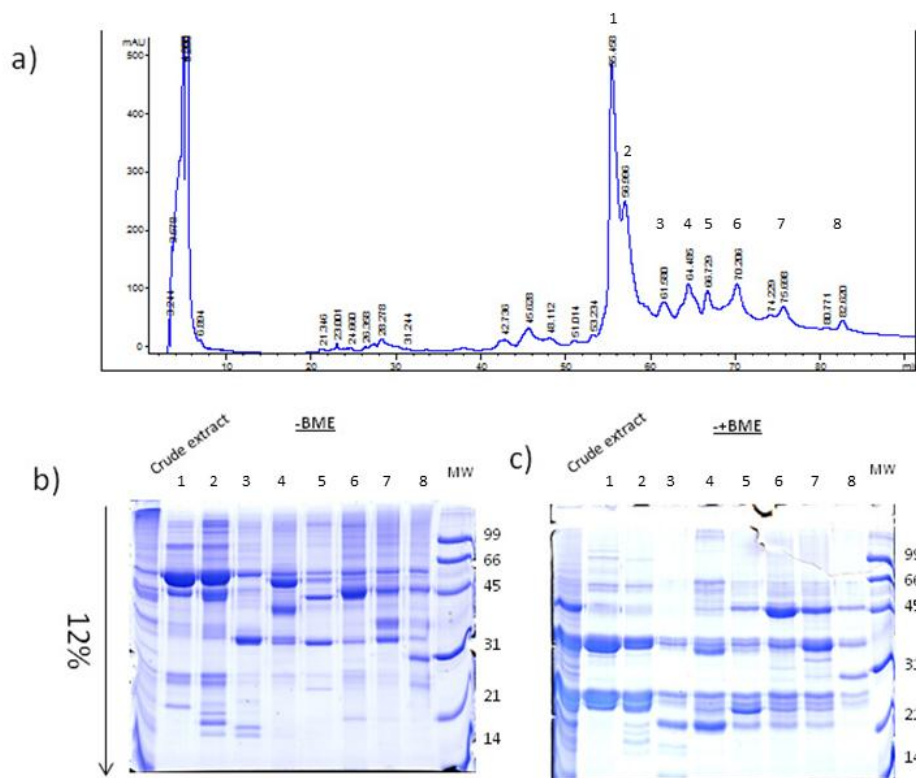


Figure 12. (panel a) C8 RP HPLC chromatographic separation of hazelnut proteins; R250 stained 1D PAGE of collected peak (indicated with progressive numbers) treated in absence (-BME) (panel b) and in presence (+BME) (panel c) of  $\beta$ -mercaptoethanol as reducing agent.

When the chromatographic separation was carried out with the crude extract pretreated with reducing agents (10 mM DTT, 67 °C, 1 hour) (Figure 13) a new cluster of polar components, eluted at the beginning of the chromatogram, was detected and the MALDI-ToF mass spectrometry analysis allowed the identification of several fragment of high molecular weight proteins, released after disulfide bonds reduction, and the presence of the *ns*-lipid transfer protein. Moreover two new peaks related to the two chains of 11S globulin like proteins have been also detected.

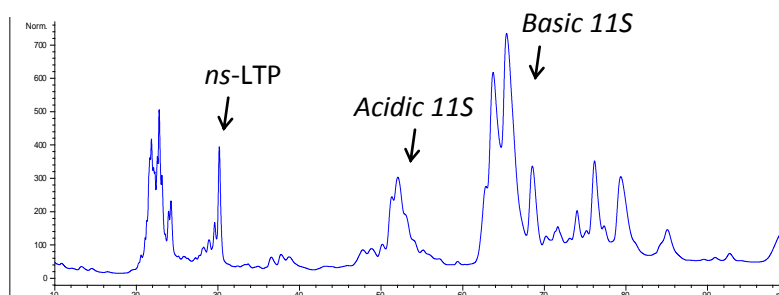


Figure 13. C8 RP HPLC chromatographic separation of hazelnut proteins pretreated with reducing agent (10 mM DTT, 67 °C, 1 hour)

### 2.2.3.4 Shotgun proteomic analysis

Protein mixtures were reduced/alkylated, and the tryptic digest was analyzed by nanoHPLC/ESI-MS/MS. The gel free shotgun proteomic approach allowed the detection of new hazelnut proteins (Table 1) through the identification of homologous gene products indexed for other seeds, with relatively low amino acid coverage values.

DB	Accession	score	Mass Da	Matches (unique)	Description
NCBIInr	gi 22135427	158	62814	3(3)	Castanin <i>Castanea crenata</i>
NCBIInr	gi 118340979	70	56865	3 (3)	7S globulin <i>Ficus pumila</i>
SwissProt	HSP7B_ARATH	48	71741	1(1)	Heat shock 70 kDa <i>Arabidopsis thaliana</i>
NCBIInr	gi 388514675	44	36480	3(2)	unknown <i>Lotus japonicus</i>
NCBIInr	gi 7484767	44	57185	2(2)	protein body membrane MP27 <i>cucurbit</i>
NCBIInr	gi 1181673	43	22537	1(1)	Heat shock 70 kDa <i>Sorghum bicolor</i>
NCBIInr	gi 224126933	27	52898	1(1)	Predicted protein <i>Populus trichocarpa</i>
SwissProt	11S2_SESIN	27	52083	4(2)	11S globulin 20S <i>Sesamum indicum</i>

**Table1.** Summary of Mascot Search results related to the shotgun of total hazelnut extracted proteins. In the table are reported all the proteins identified by homology. Major storage proteins already identified by the 2D-MS approach were omitted.

## 2.3 Characterization of N-glycans of the 48 kDa glycoprotein, Cor a 11 by hydrophilic interaction liquid chromatography and MALDI-TOF MS

The definition of both the specific-site of glycosylation and the glycan structure is crucial to elucidate the pattern of cross-reactivity of the protein and to make more sensible and accurate the diagnosis and the therapy.

### 2.3.1 Materials

Recombinant PNGase F from *Flavobacterium meningosepticum* (PNGase F), zwitterionic-HILIC material (ZICHILIC™, 200 Å, 10 mm, zwitterionic sulfobetaine functional groups (from SeQuant AB, Umeå, Sweden)), GELoader tips from Eppendorf. POROS 10 R2 reversed-phase chromatography medium was obtained from PerSeptive Biosystems.

### 2.3.2 Methods

#### 2.3.2.1 HILIC glycopeptides enrichment

The Cor a 11 spot was excised from the 2D-PAGE and subjected to “in gel” trypsin digestion as previously described. Peptides were dried down in a Speed Vac centrifuge and desalted onto ZipTip C18 Pipette tips (Millipore). The GELoader pipette tip were carefully flattened near the end of the outlet. The microcolumn was plugged by tiny pieces of C8 resin (3M, Milan, Italy) opportunely positioned at the lower end and packed with zwitterionic-HILIC material as described by Gobom et al. 1999. Prior to using the column, a washing step was performed with 20 µL of ACN 0.1 % TFA (v/v), followed by an equilibration step with 20 µL of washing buffer 0.5% (v/v) TFA. The peptide sample was lyophilized and dissolved in 10 µL of washing buffer and applied to the HILIC microcolumn, washed twice with 20 µL of ACN 0.1 % TFA (v/v), (collecting flow-through solutions) and eluted in two steps with 8 µL washing solution and 8 µL of ACN 0.1 % TFA (v/v). Eluate was collected in a clean tube and a dried aliquot (0.5 µL) redissolved in 50% ACN/1% FA was used for MALDI-TOF MS analysis (Gobom et al., 1999).

#### 2.3.2.2 Deglycosylation

The HILIC enriched fraction was dried and redissolved in 100 mM AMBIC (10 µL), pH 7.8, and incubated with 1 mU PNGase F at 37°C overnight. The sample was desalted trough a Poros R2 resin (Applied Biosystems, Framingham, MA) microcolumn prepared as described by Gobom et al 1999. A suspension of chromatography medium in propan-2-ol was deposited into the GELoader pipette tip and gently pressed through, thus allowing the chromatography medium to form a column of approximately 1.5 mm. Deglycosylated peptides were applied to the microcolumn, washed with 0.5% FA, and eluted with 0.5-1 µL of 70% ACN/1% FA for direct transfer to a MALDI TOF-MS target.



### 2.3.2.3 MALDI-TOF analysis

MALDI-TOF MS spectra were acquired by a Voyager STR-DE Pro mass spectrometer (Applied Biosystems) equipped with delayed extraction technology. Mass spectra were acquired both in positive linear or reflector mode and 10 mg/mL DHB dissolved in 1% FA or CHCA in 50% ACN/ 0.5% FA were used as matrices for the analysis of glycopeptides and deglycosylated peptides, respectively.

### 2.3.3 Results and discussion

Asn<sup>254</sup> is the only glycosylated site already described in literature for the Cor a 11 (Schocker et al., 2004; Muller et al., 2000). MALDI-TOF analysis of the HILIC enriched glycopeptides (Figure 14) indicated that actually both of the potential glycosylation site of the Cor a 11 sequence, Asn<sup>254</sup> and Asn<sup>38</sup>, are actually glycosylated. The glycopeptides map verified the presence of a xylosylated high-mannose glycan linked to the Asn<sup>254</sup>, in agreement with previous studies (Lauer et al., 2004), and a high-mannose glycan linked to the Asn<sup>38</sup>.

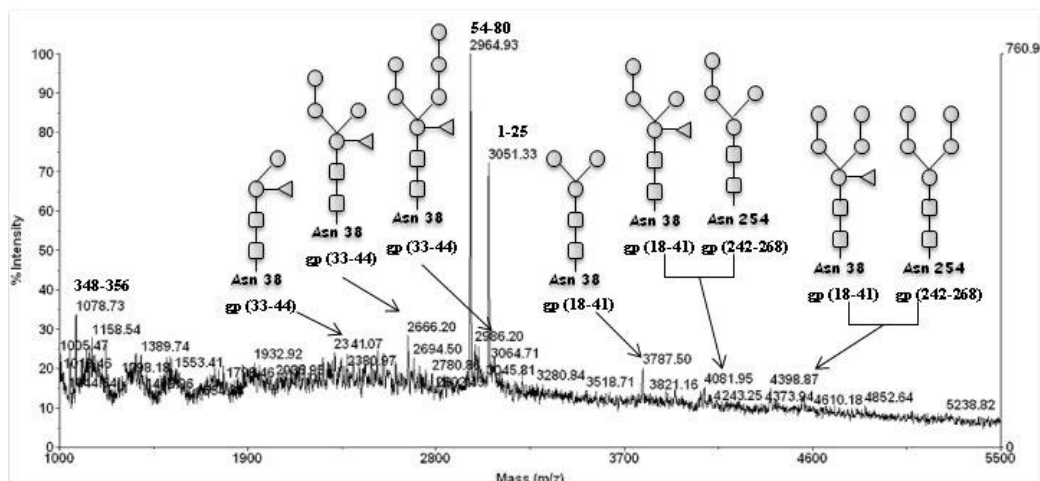


Figure 14. MALDI-ToF MS spectra of N-linked tryptic glycopeptides of Cor a 11 selectively enriched with a microscale chromatographic step using the HILIC – Glycan structure: ○ Man, □ GlcNAc, △ Xyl.

More in deep, the signals in the mass range of 4081.95 – 4398.87 were assigned to Asn<sup>254</sup> glycosylated peptides on the basis of the characteristic mass loss of the glycan moiety, i.e., hexose (Hex) – 162 units, N-acetyl glucosamine (GlcNAc) – 203 units and N-acetylneuraminic Xylose (Xyl) – 132 units (Figure 14). The signals in the mass range of 2341.07 – 2986.20 and 3787.50 – 4398.87 were assigned to 2 series of Asn<sup>38</sup> glycosylated peptides belonging to different trypsin cleavage sites (Figure 14). Other signals in the spectrum were assigned as aspecific non glycosylated peptides which are regularly observed to be co-enriched with the glycopeptides for their high hydrophilicity. MALDI-TOF analysis of the deglycosylated peptides confirmed both glycosylation sites and the glycan structures hypothesized (Figure 15).

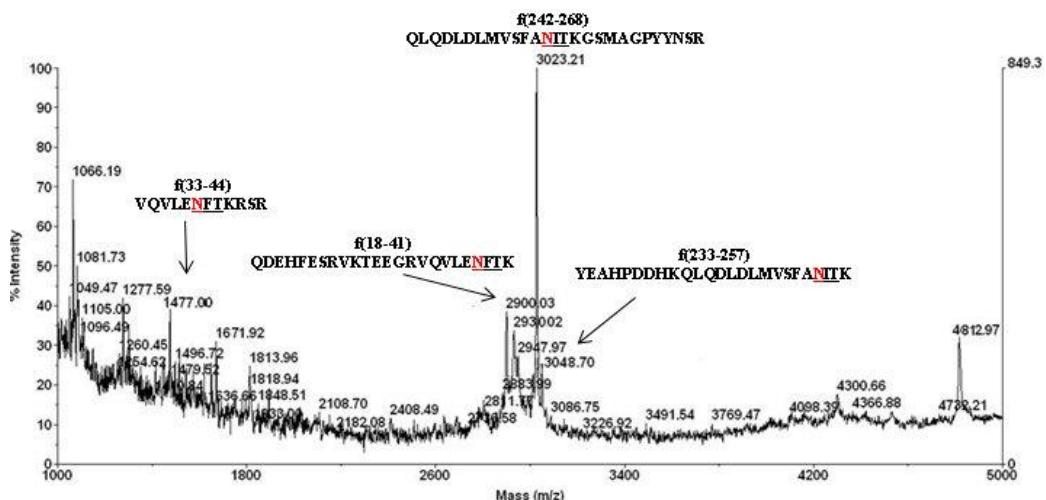


Figure 15. MALDI-ToF MS spectra deglycosylated peptides

Deglycosylation drastically improved peptide detection while linker Asn residues were converted into aspartic acid with a 1-Da mass unit shift (Gonzalez et al., 1992). The signals at  $m/z = 1477.00$  and  $2900.03$ , well matches respectively with the tryptic Cor a 11 (33-48) and (18-41) peptides carrying the xylosylated high-mannose oligosaccharide chain. The strong signals at  $m/z = 3023.21$  and  $2930.02$  corresponds with the tryptic Cor a 11 (242-268) and (233-257) carrying the high-mannose oligosaccharide chain.

## **2.4 Purification and characterization of *non-specific* Lipid Transfer Protein (*ns*-LTP), Cor a 8. (IFR- Institute of Food Research).**

The *ns*-LTP represents up to the 4% of the extract and may be missing from some extract preparation inducing a low diagnostic sensitivity (Hoffmann-Sommergruber et al., 2008). The development of optimized methods for purification of allergenic molecules is the basis not only for the study of biological and immunological characteristics (Pastorello et al., 2001) but also for the development of more sensitive and specific diagnostic tests. The purification of allergens from food instead to produce it in recombinant form has two advantages: (i) the post-translational modifications, which play a fundamental role in the biological activity of the IgE epitope structures, are preserved and (ii) isoforms of the same protein are together purified, replicating the composition of the food more closely is possible (Hoffmann-Sommergruber et al., 2008). An optimized protocol for *ns*-LTP protein purification was performed slightly modifying the method published by Rigby et al. 2008.

### **2.4.1 Materials**

Italian and Turkish raw hazelnut cultivars were used. Rabbit polyclonal antibody anti-rCor a 8 (Phadia, Uppsala, Sweden); Goat anti-rabbit IgG (whole molecule) alkaline phosphatase conjugate (Sigma-Aldrich, Dorset, UK). Gels, reagents and protocols for analytical SDS-PAGE were from Invitrogen, Renfrewshire, UK and for immunoblotting were from Bio-Rad, Hertfordshire, UK. All other chemicals were of AR grade unless specified and were obtained from Sigma-Aldrich, unless specified.

### **2.4.2 Methods**

#### **2.4.2.1 Preparation of protein extract for immunoblotting analysis**

Shelled raw hazelnuts and the hazelnuts manually deprived of husk were ground in liquid nitrogen using a Waring Blendor (Fisher Scientific, Leicestershire, UK) and defatted by stirring in five volumes (w/v) of hexane for 1 h at room temperature. After drying, the flour was reground in an electronic grinder and the hexane extraction repeated. The hazelnut husk was ground in an electronic coffee grinder, separately. Proteins were extracted starting from 50 mg of flour dissolved 1 mL of extraction buffer (25% NuPAGE LDS sample buffer (4X), 65% water, 10% 0.5M DTT), mixed on a wheel mix for 1h at room temperature. Samples were heated for 10 minutes at 70°C and centrifuged for 3 minutes at 10000 rpm.

#### **2.4.2.2 SDS-PAGE analysis**

SDS-PAGE was performed on 4–12% gradient gels run for 35 min at 200 V in MES buffer, reduced gels were run by including 50 mM 1,4-dithioerythritol in the denaturing heating step. Reference markers with known molecular weights (Mark 12 unstained standard, Invitrogen) were run on the same gel. Gels were fixed in methanol: water: acetic acid (40:50:10 v/v) and stained using colloidal Coomassie G250 (Invitrogen). Gels were scanned using a GS800 calibrated densitometer and Quantity One software (Bio-Rad).

#### **2.4.2.3 Immunoblotting analysis with anti rCor a 8**

Gels for immunoblotting were run as above except the molecular weight standard was replaced by one containing pre-stained molecular weight marker proteins (SeeBlue Plus2, Invitrogen). Immunoblotting was performed using semi-dry blotting of proteins from gels to NC membrane (0.2- $\mu$ m pore, Trans-Blot transfer media, Bio-Rad) in 39 mM glycine, 48 mM Tris base, 0.0375% SDS, 20% methanol, pH 8.8–8.9 using a Trans-Blot SD, semi-dry transfer cell (Bio-Rad) at 15 V for 20 min. Membranes were blocked by incubation (1 h, at room temperature) in 5% skim milk powder (Marvel, Premier Foods, Lincolnshire, UK) in TBST (0.05M Tris, 0.138M NaCl, 0.0027M KCl, 0.05% v/v Tween 20, pH 8.0) and washed three times in TBST (15 min, 50 mL). The membrane was incubated using gentle shaking in a 1:10000 dilution of anti-rCor a 8 for 1 h at room temperature and then washed in TBST (as above). The blot was then incubated with gentle shaking in a 1:10000 dilution of goat anti-rabbit alkaline phosphatase conjugate for 3 h at room temperature and then washed in TBST (as above). Bound antibody was located by briefly washing the membrane in water (30 min, 100 mL) and then stained using 10 mL of a SIGMA FAST™ BCIP/NBT substrate tablet solution (1 tablet in 10 mL of water). The washing step is sufficient to remove any inhibitory effect of phosphate on the alkaline phosphatase.



#### 2.4.2.4 Purification of *ns*-LTP

Proteins were extracted as described by Rigby et al. 2008, by stirring 60 g of flour in 800 mL of 20 mM MES, pH 5.6 (1:13, w/v) containing 6.2 mM sodium azide, 3% (w/v) polyvinylpyrrolidone (PVPP, Sigma Aldrich), which adsorb phenolic compounds, and protease inhibitor (4 tablets, Roche Complete Protease inhibitor tablet) for 30 min at room temperature. The solution was centrifuged (4000 x g, 30 min, 10°C), the supernatant was filtrated through a coarse glass sinter and the pH was adjusted at pH 6 by adding HCl and diluted with 150 mL of PBST. The solution of proteins was loaded into a cation exchange column (2.6x15 cm) of STREAMLINE SP XL (GE Healthcare, Life Sciences) manually packed. The column was equilibrated and washed with 20 mM MES (pH 6) to remove unbound molecules. Isocratic elution was realized using 20 mM MES (pH 6), 1M NaCl buffer, controlled with a low-pressure peristaltic pump. The manually collected fractions, that showed absorbance at 280 nm, were pooled together. All chromatographic separations were conducted on a BioCad Sprint HPLC system (Applied Biosystem), using UNICOR 5.01 software. Fractions were monitored at 220 and 280 nm since the *ns*-LTP have low content of aromatic amino acids and therefore detection at 280 nm was not sufficiently sensitive. Proteins solution was ultrafiltered using a regenerated cellulose membrane with a 1-kDa exclusion limit (Millipore, Hertfordshire, UK), ammonium sulphate was added to get the final concentration of the solution 3M and the solution was centrifuged (4000 x g, 30 min 20°C) before applying the supernatant to the column. The efficiency of separation for two hydrophobic chromatographic interaction columns was tested. The high density POROS® HP2, (0.4x10 cm, Applied Biosystems) was compared with the (16 x 130) Phenyl Sepharose high substitution (GE Healthcare) column used in the published method. In both case columns were equilibrated in 15 mM MES, 3M ammonium sulphate, pH 6 (Buffer B). Cor a 8 was eluted with a decreasing ammonium sulphate gradient (3.0 to 0.0 M in equilibration buffer over 180 mL in equilibration buffer at 3mL/min). Fraction containing Cor a 8 were identified by immunoblotting with anti-rCor a 8, pooled and concentrated by ultrafiltration and dialyzed against distilled water (1h, 1L) using 3.5 kDa cut-off dialysis tubing (Perbio Science, Northumberland, UK) and re-concentrated by ultrafiltration (1-kDa membrane). The last step of purification was a cation-exchange chromatography. MES was added to obtain 40 mM final concentration, the pH was adjusted to pH 5.6 and the solution was applied to a Poros HS20 (0.46x10 cm) (Applied Biosystem) equilibrated with 20 mM MES, pH 5.6. Proteins were eluted with 0 – 0.5 M NaCl gradient.

#### 2.4.3 Results and discussion

The *ns*-LTP is known to be essentially concentrated in the pericarp of the *Rosaceae* fruit types whereas the pulp contains lower amounts of allergens (Borges JP et al., 2006). Proteins extracted from husk, hazelnut with and without husk were separated by 1D electrophoresis (Figure 16, panel a). The image analysis revealed similar protein profiles for both the hazelnut with and without husk while for the husk material shows the presence of a less amount of proteins all concentrated in a range of molecular weight lower than 30 kDa. Although the presence of the *ns*-LTP was confirmed by mass spectrometry analysis for all of the samples analyzed, antibodies directed against the recombinant Cor a 8 detected the protein only in proteins extracted from hazelnuts deprived of the husk (Figure 16, panel b). The discrepancies of these results may be due to the high concentration of polyphenols in the husk which may affect both the extraction and/or the immunogenic capacity of the allergen, by binding the surface of the protein (Del Rio et L., 2011).

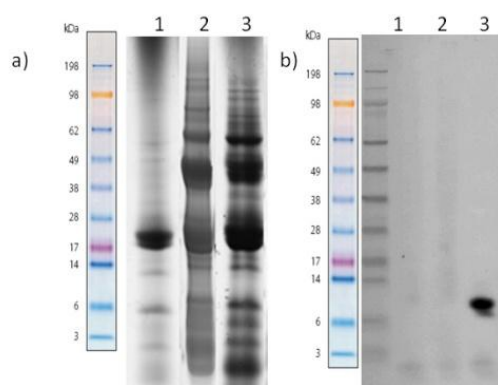


Figure 16. ( panel a) CBB R250 NuPAGE 4-12% Bis-Tris Gel of extracted proteins form husk (line 1), nut with husk (line 2), nut without husk (line 3),(panel b) Western blotting stained with anti rCor a 8.

Cor a 8 was purified from raw hazelnuts in three steps of purification slightly modifying the method by Rigby et al., 2008. The first step of purification was an expanded bed adsorption chromatography which captures proteins directly from unclarified crude sample whilst all the biological material suspension passes through. As the bed expands, it increases adsorbent surface contact, making interaction with the targeted molecules more effective (Amersham Pharmacia Biotech. 1997; Fernandez-Lahore et al., 2001; Roy et al., 199). The efficiency of this technique resulted in a short process time, high process yield, and limited proteolytic degradation of the target

proteins. When the protein extract was fractionated with a Phenyl Sepharose column as described by Rigby et al. 2008 (Figure 17, panel b) the *ns*-LTP was eluted over a broad range (Rigby et al 2008). Otherwise the high density Phenyl Sepharose column HP20 allowed the elution of the *ns*-LTP in a single sharp peak, labelled with a filled star in the figure 17 (panel b), as confirmed by western blotting analysis of collected fractions blotted with anti-rCor a 8 IgE antibody (Figure 17). Both the SDS gel analysis and the western blotting of the eluted protein showed the presence of a single band of about 7 kDa (Figure 17).

The last step of purification was a cation-exchange chromatography (Figure 18). According with the western blot analysis the *ns*-LTP represented only a very small portion of the hydrophobic interaction chromatographic fraction. The protein was eluted into two peaks. Peptide mass fingerprinting by MALDI-MS analysis of the main peak did not allowed any identification of the protein, probably because the sequence is missed in both genomic and proteomic on line databases.

The RP-HPLC analysis and the size-exclusion chromatography confirmed the purity and the range of expected molecular weight of the *ns*-LTP, respectively. The peptide mass fingerprinting analysis of the in gel tryptic digest further confirmed the identified of the *ns*-LTP (score=553) with sequence coverage of 72%. The improvement of the chromatographic separation firstly by applying the “capturing” chromatography followed by the use of a HP20 column allowed obtaining a higher yield of purification by a less number of steps of fractionation.

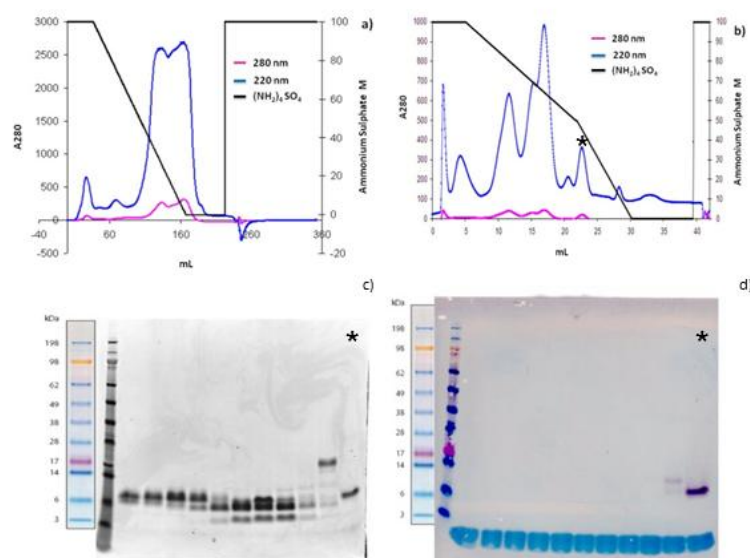


Figure 17. Crude extract from preparative chromatography was applied to two different hydrophobic interaction chromatography on a column Phenyl Sepharose high substitution column (panel a) POROS® HP2 (panel b). The POROS HP allowed the purification of the *ns*-LTP in a sharp peak, labelled with a filled star, according with the CBB R250 SDS-PAGE (panel c) and the relative western blotting stained with rabbit polyclonal anti-rCor a 8 anti-serum (panel d).

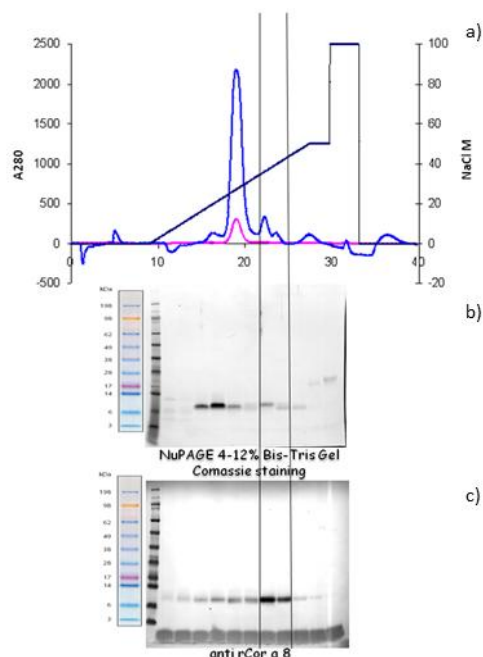


Figure 18. (panel a) cation exchange chromatography. The *ns*-LTP was eluted in two peak indicated with two lines, (panel b) CBB R250 SDS-PAGE of the collected peak and (panel c) corresponding relative western blotting stained with rabbit polyclonal anti-rCor a 8 anti-serum.

## 2.5 Conclusion

The development of new high-resolution separation and enrichment techniques has been applied successfully to seed storage proteome characterization providing a dramatic improvement of results in database searching by increasing the confidence in protein identifications and the coverage of proteomes.

The application of the size exclusion chromatography for removing the globulines, the high-abundance seeds storage proteins, and for enriching of the low-abundance proteins, enhanced efficiently the identification and the characterization of new allergens.

The shotgun analysis allowed overcoming another typical obstacle that occurs in seeds storage proteome characterization of not availability of full genomic and proteomic sequences databases for many species. By this way, in fact, has been possible to identified not yet indexed hazelnut proteins by homology gene products arising from other seeds, though with a relatively low sequence coverage.

Since the glycopeptides represent only a minor portion of the peptide mixture and their signals are easily suppressed by those of the coexisting, strongly ionizing, non-glycopeptides in spectrometry analysis, the hydrophilic interaction chromatography (HILIC) technique (Gobom et al., 2008), which exploits the hydrophilicity of the glycans of glycopeptides to separate from the total peptides pool and enrich it, well makes up this deficiencies. These evidences properly explained in literature why is missed a clear and complete characterization of the N-glycan structure of Cor a 11. In fact, by applying the HILIC has been possible confirmed the effective glycosylation of both potential site of the Cor a 11 and characterized the glycan structures.

A new method for ns LTP purification has been developed. The ns-LTP is one of the major allergens responsible for long term sensitization in adulthood.

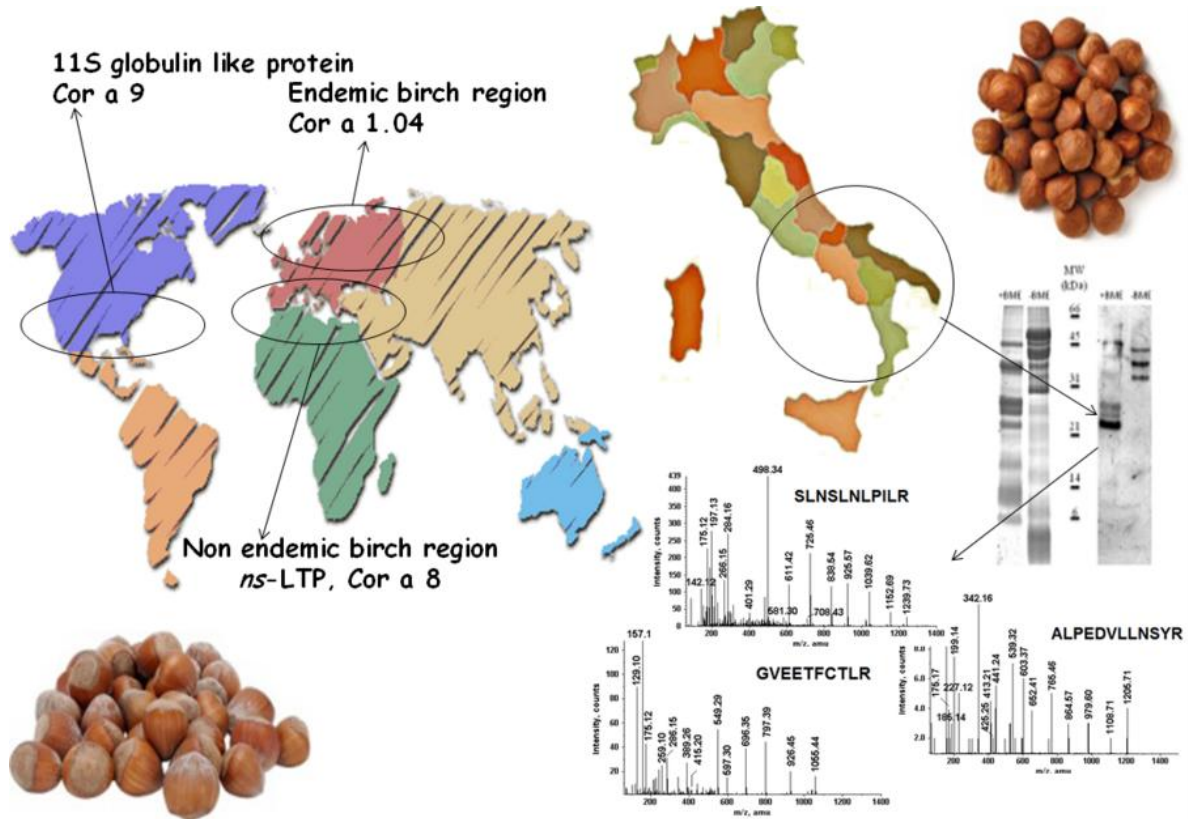
The addressing of high-throughput liquid chromatography/mass spectrometry methods for detection of hidden allergens need the availability of full genomic and proteomic in on line database used for MS/MS data processing. The addressing of new proteomic approach to hazelnut proteome study allowed a more complete definition of proteome and the definition of markers for assessment of quality control of final products.

## 2.6 References

- Amersham Pharmacia Biotech (1997) EBA Handbook: Principles and Methods. Uppsala, IBSN 91-630- 5519-8, p160.
- Borges JP, Jauneau A, Brulé C, Culerrier R, Barre A, Didier A, Rougé P (2006) The lipid transfer proteins (LTP) essentially concentrate in the skin of *Rosaceae* fruits as cell surface exposed allergens. *Plant Physiol Biochem* 44(10):535-42.
- Del Rio D, Calani L, Dall'Asta M, Brighenti F (2011) Polyphenolic composition of hazelnut skin. *J Agric Food Chem* 59(18):9935-41.
- Fernandez-Lahore HM, Lin DQ, Hubbuch JJ, Kula MR Thommes J (2001) The use of ionselective electrodes for evaluating residence time distributions in expanded bed adsorption systems. *Biotechnology Progress* 17:1128-1136
- Garino C, Zuidmeer L, Marsh J, Lovegrove A, Morati M, Versteeg S, Schilte P, Shewry P, Arlorio M, van Ree R (2010) Isolation, cloning, and characterization of the 2S albumin: a new allergen from hazelnut. *Mol Nutr Food Res* 54(9):1257-65.
- Gobom J, Nordhoff E, Mirgorodskaya E, Ekman R, Roepstorff P (1999) Sample purification and preparation technique based on *nano*-scale reversed-phase columns for the sensitive analysis of complex peptide mixtures by matrix assisted laser desorption/ionization mass spectrometry. *J Mass Spectrom* 34:105–116.
- Gonzalez J, Takao T, Hori H, Besada V, Rodriguez R, Padron G, Shimonishi Y (1992) A method for determination of N-glycosylation sites in glycoproteins by collision-induced dissociation analysis in fast atom bombardment mass spectrometry: Identification of the positions of carbohydrate-linked asparagine in recombinant alpha-amylase by treatment with peptide-N-glycosidase F in 18O-labeled water. *Anal Biochem* 205:151–158.
- Hoffmann-Sommergruber K, Mills EN, Vieths S (2008) Coordinated and standardized production, purification and characterization of natural and recombinant food allergens to establish a food allergen library. *Mol Nutr Food Res*. 52 Suppl 2:S159-65.
- Lauer I, Foetisch K, Kolarich D, Ballmer-Weber BK, Conti A, Altmann F, Vieths S, Scheurer S (2004) Hazelnut (*Corylus avellana*) vicilin Cor a 11: molecular characterization of a glycoprotein and its allergenic activity. *Biochem J* 383:327–334.
- Moreno FJ, Clemente A (2008) 2S Albumin Storage Proteins: What Makes them Food Allergens? *Open Biochem J* 2: 16–28.
- Müller U, Lüttkopf D, Hoffmann A, Petersen A, Becker WM, Schocker F, Niggemann B, Altmann F, Kolarich D, Haustein D, Vieths S (2000) Allergens in raw and roasted hazelnuts (*Corylus avellana*) and their cross-reactivity to pollen. *European Food Research and Technology* 212(1): 2-12.
- Natarajan SS, Krishnan HB, Lakshman S, Garrett WM (2009) An efficient extraction method to enhance analysis of low abundant proteins from soybean seed. *Anal Biochem* 394(2):259-68.
- Pastorello EA, Trambaioli C (2001) Isolation of food allergens. *J Chromatogr B* 756:71 –84.
- Rigby NM, Marsh J, Sancho AI, Wellner K, Akkerdaas J, van Ree R, Knulst A, Fernández-Rivas M, Brettlova V, Schilte PP, Summer C, Pumphrey R, Shewry PR, Mills EN (2008) The purification and characterisation of allergenic hazelnut seed proteins. *Mol Nutr Food Res* 52 Suppl 2:S251-61.
- Roux KH, Teuber SS, Sathe SK (2003) Tree Nut Allergens. *Int Arch Allergy Immunol* 131:234–244.
- Roy I, Pai A, Lali A, Gupta MN (1999) Comparison of batch, packed bed and expanded bed purification of *A. niger* cellulase using cellulose beads. *Bioseparation* 8:317-326.
- Schocker F, Lüttkopf D, Scheurer S, Petersen A, Cisteró-Bahima A, Enrique E, San Miguel-Moncín M, Akkerdaas J, van Ree R, Vieths S, Becker WM (2004) Recombinant lipid transfer protein Cor a 8 from hazelnut: a new tool for in vitro diagnosis of potentially severe hazelnut allergy. *J Allergy Clin Immunol* 113(1):141-7.

### 3.

## Section 2: Proteomic characterization of the alkaline subunit of a hazelnut (*Corylus avellana*) 11S globulin isoform, a new putative food allergen



### 3.1 Aim of the study

The complexity of the hazelnut allergen pattern challenges a clear definition of both the allergens and the allergenic determinants (epitopes). Therefore, it is still missing for hazelnut a program of allergen standardization in order to support innovative diagnostic and therapeutic approaches. Over the years, at least five proteins have been reported as potential hazelnut allergens. The predominance of specific allergens appears to be strictly related to both the geographical origin and the age of the allergic subjects. Advanced integrated proteomic strategies coupled with immunochemical assays were exploited to investigate the pattern of the immunological response of 15 hazelnut allergic children from Campania region.

### 3.2 Materials

Three hazelnut cultivars from Regione Campania (Mortarella, San Giovanni, Tonda) and one from Oregon previously characterized were used.

#### 3.2.1 Patients

Sera were obtained from hazelnut allergic subjects (N = 15, 80% male), all from Regione Campania (Southern Italy). Diagnosis of IgE-mediated hazelnut allergy was confirmed using skin prick test (SPT) and an oral food challenge. Either an SPT hazelnut solution or fresh hazelnuts were applied to the patient's volar forearm. Test were performed using a 1-mm singles peak lancet (ALK, Copenhagen, Denmark), with histamine dihydrochloride (10mg/ml) and isotonic saline solution (0.9% NaCl) as the positive and negative controls, respectively. Reactions were recorded based on the largest diameter (in millimeters) of the wheal and flare at 15 min. An SPT result was considered "positive" if the wheal was 3 mm or larger, without a reaction to the negative control. The allergy symptoms ranged from urticaria to angioedema and anaphylaxis. The clinical features of the allergic individuals enrolled in this study are reported in Table 1. The total serum IgE was quantified with the ImmunoCAP System (Phadia, Uppsala, Sweden). All of the serum samples were stored at -20°C before being used. Any sensitization was regarded as positive when the total IgE was greater than 0.35 kUA/L.

Patient	Sex	Age at diagnosis (months)	Atopic risk	Skin prick test to hazelnut (>)	Total serum IgE (kU/L)	Symptoms at diagnosis	Concomitant other food allergies	Concomitant allergy to aeroallergens
1	M	18	Yes	Positive	191	Urticaria, angioedema	Yes	Yes
2	F	60	Yes	Positive	94,2	Angioedema, anaphylaxis	Yes	Yes
3	M	24	Yes	Positive	478	Urticaria	Yes	No
4	F	54	Yes	Positive	18,5	Angioedema	Yes	No
5	M	204	No	Positive	75,7	Urticaria	Yes	No
6	M	75	Yes	Positive	355	Urticaria	Yes	Yes
7	F	23	Yes	Positive	878	Angioedema	Yes	No
8	M	6	No	Positive	1417	Urticaria, angioedema, anaphylaxis	Yes	No
9	M	6	Yes	Positive	1378	Urticaria, angioedema	Yes	Yes
10	M	60	Yes	Positive	229	Urticaria	Yes	Yes
11	M	48	Yes	Positive	153	Urticaria	Yes	No
12	M	44	No	Positive	162	Urticaria	Yes	Yes
13	M	36	Yes	Positive	581	Angioedema	Yes	No
14	M	12	Yes	Positive	948	Urticaria	Yes	No
15	M	24	Yes	Positive	564	Urticaria, angioedema	Yes	No

**Table 2.** Clinical features of the hazelnut-allergic patients enrolled in the study.

### 3.3 Methods

#### 3.3.1 Immunoblot for IgE-binding assay and immunoblot inhibition

Both 1DE and 2DE gels were electroblotted onto nitrocellulose paper using a Trans-Blot Cell from BioRad (BioRad Laboratories, Hercules, CA, USA) at 400 mA at 4 °C for 1 h. Membranes were blocked for 1 h at room temperature with (5% w/v) bovine serum albumin (Sigma) in Tris-buffered saline solution with 0.05% Tween 20 (TBS-T) and incubated overnight at 4 °C, with the sera of allergic children or control individuals (N=3). Several combinations of hazelnut protein amounts and serum dilutions (in TBS-T) were tested to minimize the non-specific antibody response. The 2DE blot was immunostained with 200 µg of pooled sera in 40 mL of TBS-T. Immunoblot inhibition experiments were performed by pre-incubating a serum (1 h at room temperature) with 2 µg of the

purified ~21 kDa IgE-reactive protein. After washing with TBS-T, monoclonal peroxidase-conjugated mouse anti-human IgE antibody (Sigma) diluted in blocking solution (1/10,000) was applied to the membrane for 1 h at room temperature. The membrane was extensively rinsed with TBS-T (3×10 min) and finally with TBS (1×10 min) before development. Chemiluminescence reagents (ECL Plus WB reagent, GE Healthcare) and X-ray film (Kodak, Châlons/Saône, France) were used to visualize the immunoreactive protein bands at various exposure times ranging from 0.5 to 10 min.

### 3.3.2 Purification of the IgE-binding protein

The predominant IgE-binding protein was purified by a sequential two-step procedure. The first step was size-exclusion chromatography on a Superdex 75 column, previously calibrated with a standard protein mixture (Amersham), bovine serum albumin (BSA, Mw 66 kDa) and ribonuclease A (RNase A, Mw 13.7). Proteins were eluted with 50 mM sodium phosphate buffer (pH 7.5) and 150 mM NaCl. The second step of purification was Reverse Phase (RP) – HPLC using a C<sub>8</sub> Vydac 2.1 mm i.d. column (Hesperia, CA, USA). After 10 min of isocratic elution using 25% solvent B (0.1% TFA in acetonitrile, v/v), a fractionated step was applied: 25-30% B for 5 min, 30-35% B for 30 min, 35-50% B for 60 min, 50-55% B for 15 min at a flow rate of 0.200 mL/min. Solvent A was 0.1% TFA in water (v/v). In both cases, the HPLC chromatograph was an HP 1100 Agilent modular system equipped with diode array detector (Palo Alto, CA, USA). The column effluents were monitored by detection at  $\lambda$ = 220 and 280 nm. The allergen-containing fractions were monitored by western immunoblotting incubated with pooled of 15 sera. IgE-immunoreactive fractions were pooled and concentrated.

### 3.3.3 MS/MS Analysis and *de novo* Sequencing

*de novo* sequencing of the tryptic peptides was assisted by the Analyst 1.1 software (Applied BioSystems), followed by manual validation of the sequences. *De novo* generated peptide sequences were used for homology searches using the MS BLAST and MS-Pattern (Protein Prospector) algorithms against the NCBI non-redundant database using standard settings with no taxonomical restriction.

## 3.4 Results and discussion

The IgE-reactivity of raw hazelnut proteins was tested by immunoblotting using sera from 15 pediatric patients affected by hazelnut allergy. To rule out the possibility that the immune-reactive patterns were associated with specific hazelnut cultivars, experiments were performed using protein extracts from three cultivars from Southern Italy (Mortarella, San Giovanni and Tonda) and one from Oregon (USA). Firstly immunoblotting experiments were carried out using sera dilutions of 1:10 – 1:50 (Pastorello et al., 2002; Barre et al., 2008; Garino et al., 2010; Flinterman et al., 2008; Schocker et al., 2001). By this method, all of the most abundant proteins were immunostained, exhibiting clear IgE-recognition (Figure 19). Further tests with several combinations of crude hazelnut protein amounts and serum dilutions allowed for the minimization of the non-specific antibody responses. Finally, an optimal response for the most reactive protein components was obtained by applying 7  $\mu$ g of the protein extract in the electrophoretic lane and 1:200 dilution of the sera.

Figure 19. Comparison of western blotting analyses with different concentration of PBS proteins extract and dilution of sera. Line 1: 12 $\mu$ g protein - 1:100 sera dilution, line 2: 7 $\mu$ g protein - 1:100 sera dilution, line 3: 7 $\mu$ g protein - 1:200 sera dilution.

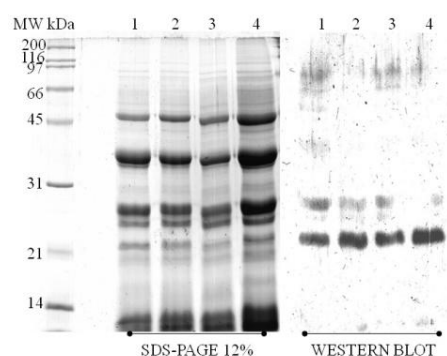


Figure 20. The IgE western blot of the extracted proteins from 3 hazelnut Campania autochthonous cultivar (1. Tonda, 2 San Giovanni, 3 Mortarella) and one Oregon cultivar (4) stained with a pool of 15 sera of hazelnut allergic children shows the immunoreactivity to a protein of about 21 kDa.



Using these conditions, all of the sera showed specific IgE reactivity against a polypeptide of approximately 21 kDa (Figure 21). A faint additional immunoreactivity to bands at 48 kDa and 9 kDa was also detected for 12 (lanes 1-2, 4-7 and 9-14 Figure 21) and 7 (lanes 1-7 Figure 21) allergic patients, respectively. The comparison by western blotting analysis of the 4 cultivars, incubated with a pool of the 15 sera, showed similar patterns of immunoreactivity among variety and the predominance of the ~21 kDa band in all cases Figure 20.

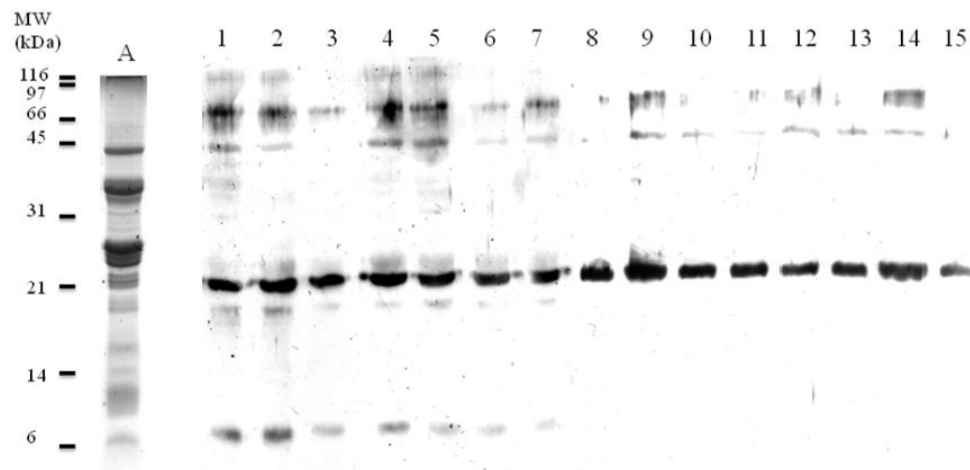


Figure 21. Western blot of PBS protein extracts from raw hazelnuts (*Mortarella Cv*) immunostained with individual sera from 15 hazelnut-allergic subjects.

The 2-dimensional (2D) Western Blot analysis against a pool of 15 sera confirmed the immunoreactivity of the protein at ~21 kDa in an 8.5-9.2 pI range (Figure 22). A faint additional IgE reactivity against bands at 48, 25 and 7 kDa was also confirmed, and the immunoreactive spots were in-gel trypsinized and identified by *nano*-LC-MS/MS. The 48 kDa band was identified as Cor a 11, the 25 kDa band was the basic chain of Cor a 9 and the 7 kDa band was Cor a 8. The IgE-affinity of the ~21 kDa spots, which quantitatively are much less represented than other storage proteins (Figure 22), was clearly the most intense.

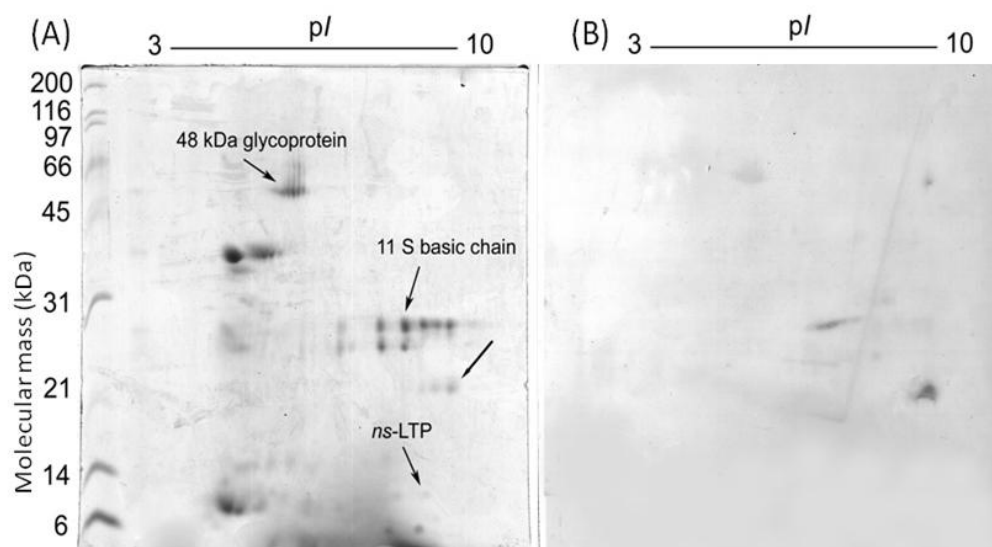


Figure 22. (panel a) 2D IEF/SDS-PAGE of PBS-extracted proteins from *Cv Mortarella* (Coomassie staining). Immunoreactive proteins were identified by MALDI-TOF MS-based Peptide Mass Fingerprinting (PMF), (panel b) corresponding immunoblot analysis with a pool of 15 sera. The predominant IgE-binding protein was the ~21 kDa/pI ~9.0-9.2 spot. Faint IgE-immunoreactivity was also detected for the 48 kDa-glycoprotein (Cor a 11), the basic chain of 11S globulin albumin-like protein (Cor a 9) and the ns-LTP (Cor a 8).



Both MALDI-TOF MS fingerprinting (Figure 23) was unsuccessful in definitively identifying the immune-reactive ~21 kDa spots, most likely because of the incomplete database annotation of the hazelnut proteome and genome.

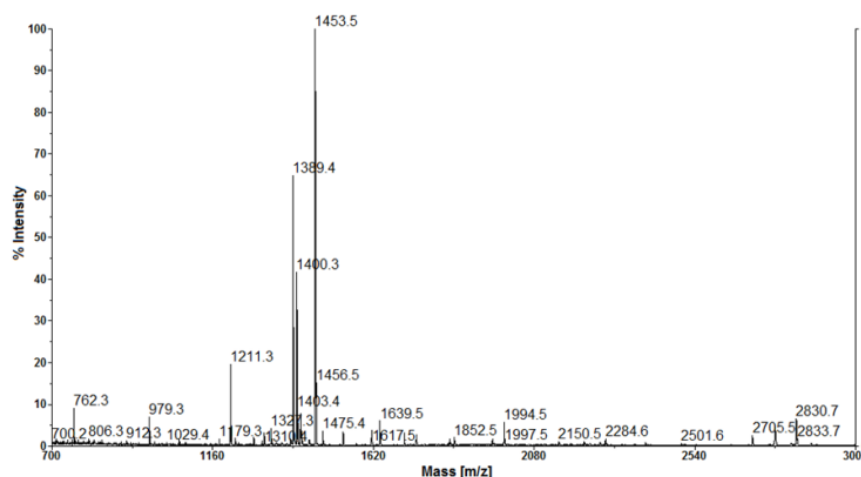


Figure 23. MALDI-Tof mass spectra of in gel trypsin digested ~21 kDa band

The hazelnut extract was also electrophoretically separated in absence of reducing agents ( $\beta$ -mercaptoethanol) and the corresponding immunoblot carried out with a pool of sera (Figure 24) showed the presence of three IgE-reactive bands with estimated MWs of ~38, 40 and 58 kDa, while the ~21 kDa IgE-reactive band was completely absent. When analyzed by MALDI-TOF MS after tryptic in-gel digestion, several peptides of the higher molecular weight bands were common to those of the ~21 kDa protein.

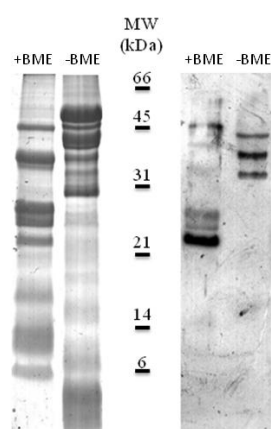


Figure 24. Western blot of hazelnut proteins separated without (-BME) and with  $\beta$ -mercaptoethanol (+BME). Three bands in the 31-45 kDa range and only one of 21 kDa were IgE reactive in the -BME and +BME conditions, respectively.

All together, these data indicated that the putative allergen is a disulfide-linked alkaline subunit of a larger protein and share structural traits with the “canonical” 11S globulin-like protein of hazelnuts (Beyer et al., 2002). The occurrence in the 2D electrophoretic gel of more than a single immunoreactive band could be attributed to a secondary N-terminal trimming subsequent to the proteolytic split of the mature protein, analogous to what has been described for the hazelnut 11S globulin (Beyer et al., 2002). Different from previous investigations about the immunogenic potential of hazelnut 11S globulin in which the acidic subunit has been described as the immunoreactive ones, we found that the IgE-reactive component was the alkaline subunit of the protein.

Despite the large number of investigations carried out to identify hazelnut allergens, the new putative allergen has never been previously described, probably because it has never been characterized at either the protein or gene level. One of the most extensive screenings of the hazelnut IgE-binding proteins was performed by Pastorello et al., 2002 who recruited 58 individuals allergic to hazelnuts from 3 Northern European centers and 7 from the Northern Italy. In this case, the immune-reactivity patterns were highly heterogeneous with no specific evidence of a ~21 kDa reactive band.

The new IgE-binding protein was purified by a sequential two-step chromatographic strategy, in order to perform a more extensive characterization. The first step was a size-exclusion chromatography on a Sephadex 75 column (Figure 25). Based on monitoring of the immunoblot (using a pool of 15 sera of allergic children) in the native chromatographic conditions, the IgE-reactive protein was eluted in a broad peak containing proteins with estimated MWs ranging from to kDa, including hazelnut 11S albumin (Cor a 9) and the 48 kDa glycoprotein (Cor a 11) (Rigby et al., 2008).

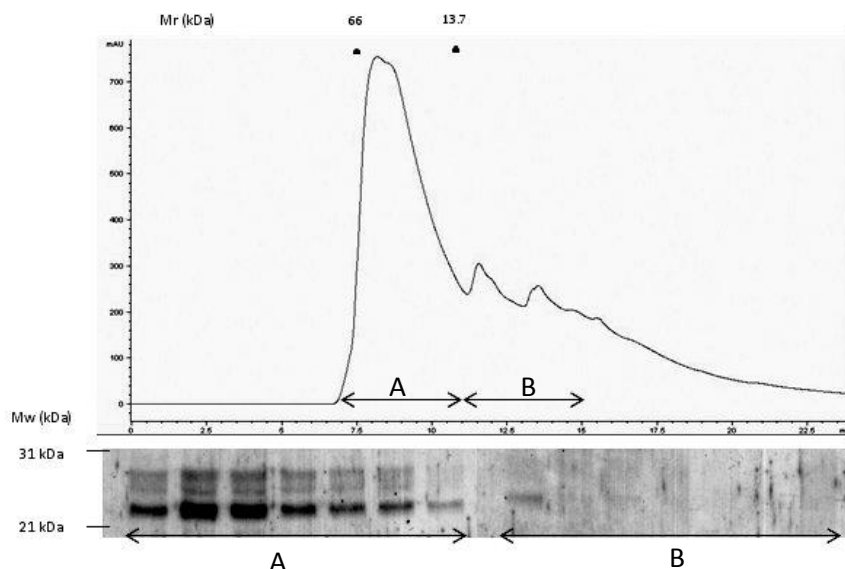


Figure 25. Gel filtration chromatography of crude hazelnut extract on a Sephadex 75 column. The fractions indicated by the double-headed arrow (A and B correspond to high and low molecular weight fractions respectively) were collected and analyzed by western blotting with sera of allergic children. The IgE-binding protein was eluted in the high molecular weight fraction with the most abundant hazelnut proteins. Molecular weight standards were indicated by filled diamonds.

A complete separation of the IgE-binding protein from the most abundant hazelnut proteins, Cor a 9 and Cor a 11, was successively achieved with a C<sub>8</sub> RP-HPLC step (Figure 26). When the collected fractions were separated by electrophoresis in presence of  $\beta$ -mercaptoethanol as reducing agents, a high number of bands were evident. Whilst, under non reducing condition, only one band of about 37-40 kDa was detected. These results are in accordance with previous findings, supporting moreover that the protein is made of more subunits bound together *via* disulphide bridges.

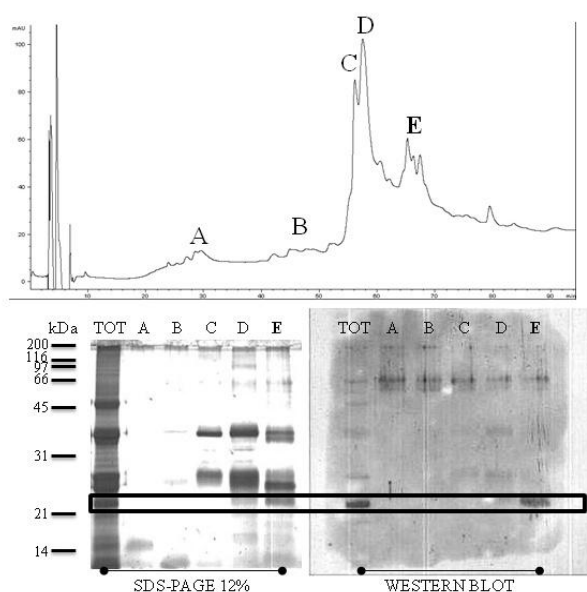


Figure 26. Partially purified protein from gel filtration was applied to a C<sub>8</sub> RP-HPLC. The peaks indicated with letters were manually collected and analyzed by 1D-SDS-PAGE and western blotting with sera of allergic children. According with blotting the IgE binding protein was eluted in the peak indicated by the letter E.

When the crude hazelnut extract was previously reduced with DTT, a single-step C<sub>8</sub> RP-HPLC analysis was sufficient to purify the ~21 kDa subunit in a sharp peak (Figure 27, panel a). MALDI-TOF MS analysis of the purified subunit showed the occurrence of a cluster of protein isoforms with MWs centered at 20.7 kDa, having a mass difference compatible with a sequential N- or C-terminus cleavage or with other post-synthetic processing events (Figure 27, panel b).

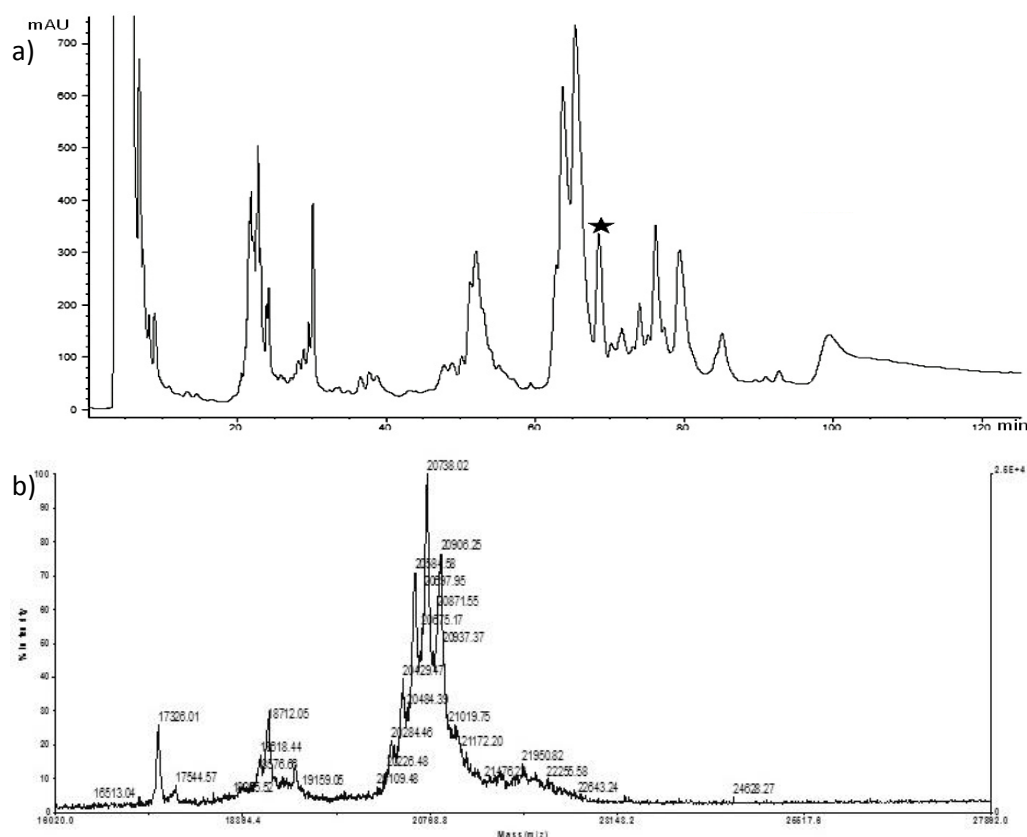


Figure 27. (panel a) RP-HPLC purification of ~21 kDa subunit from reduced crude hazelnut protein extract. The peak where the polypeptide was eluted has been indicating with a filled star, (panel b) MALDI-ToF spectra of C<sub>8</sub> RP-HPLC pure ~21 kDa subunit.

An aliquot of the pure ~21 kDa IgE-binding protein (~5 µg) was used to pre-incubate a pool of 15 sera. The immunoreactivity was completely inhibited (Figure 28) and, moreover, no other IgE-reactive bands were detected as a consequence of the signal suppression.

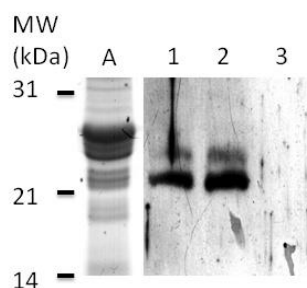


Figure 28. Western blot analysis of pure 21 kDa polypeptide (line 1) and total crude hazelnut extract (line 2) stained with a pool of 15 sera from allergic children. Inhibition assay (line 3) total crude extract was stained with a pool of sera pre-incubated with 5 µg of pure 21 kDa polypeptide (Line 3).

The subsequent nanoHPLC-ESI-Q-TOF MS/MS analysis of the tryptic digest pure ~21 kDa IgE binding subunit was unsuccessful in providing a definitive protein assignment in both the MS and MS/MS modalities of the

proteomic search engines (Mascot and Batch-Web). Whilst the manually *de novo* sequences of several tryptic peptides (Figure 29), which are listed in Table 4, were used for the identification of the protein by homology using the BLAST algorithm and the MS-Pattern tool of Protein Prospector.

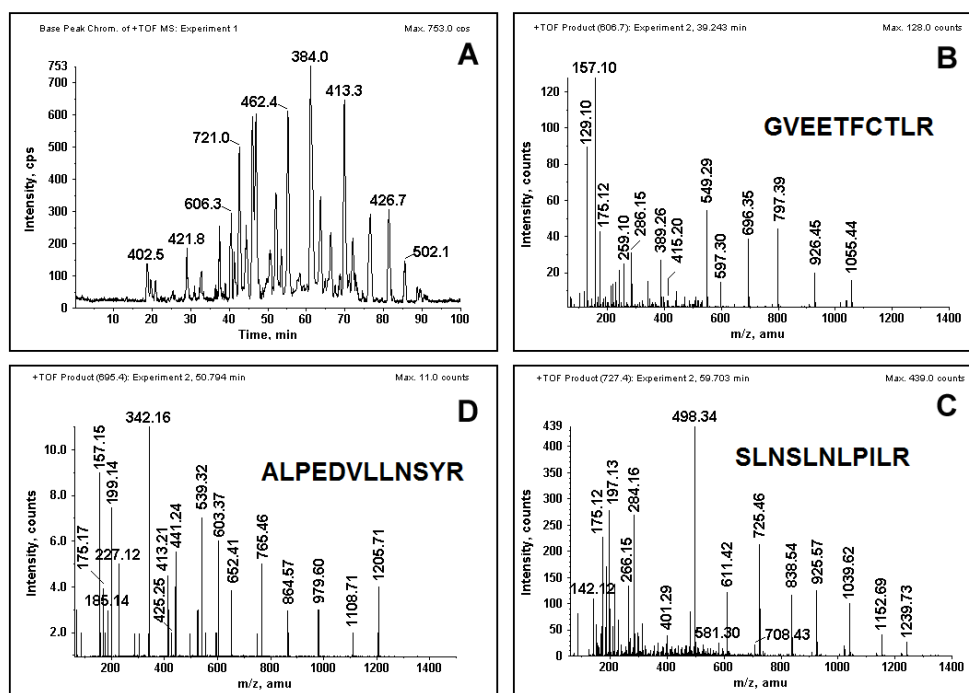


Figure 29. nano-HPLC ESI MS separation of the tryptic peptides of the 20.7 putative allergen (panel a) and MS/MS-based *de novo* sequencing of selected peptides (panel B, C and D).

Due to their identical molecular masses, the assignment of Ile or Leu in the sequences is only suggestive, and they have been designated for convenience according to the sequence of the top-ranking homologous peptides. The top-scoring proteins were isoform of 11S globulin-like proteins indexed for several plant organisms and exhibited significant or high mutual homology. The alignment of the partial sequence reconstruction of the new isoform of 11S with the “canonical” hazelnut 11S globulin shows a limited homology of sequence Figure 30.

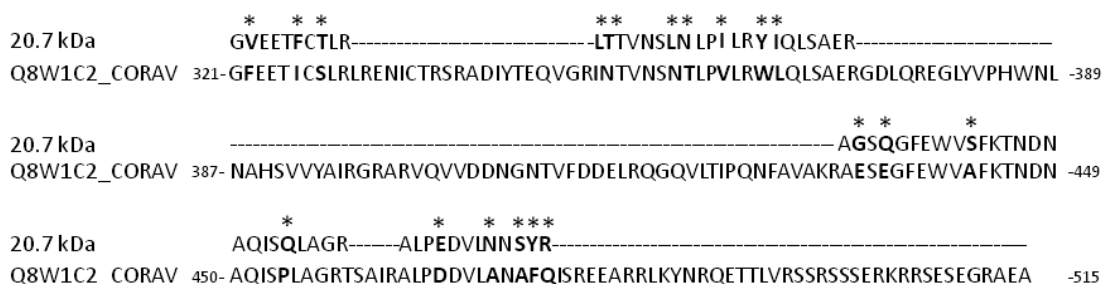


Figure 30. Alignment of the canonical hazelnut 11S basic chain with the *de novo* sequenced peptides. Amino Acid Substitutions are indicated with the asterisk.

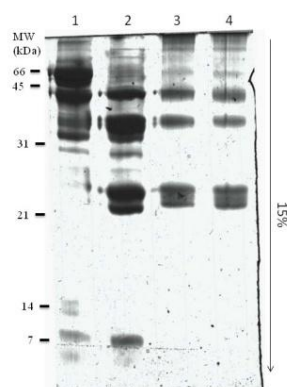
The N-terminal peptide of the alkaline subunit of the canonical hazelnut 11S globulin, arising from the endoproteolytic post-translational cleavage of the 56 kDa protein, appears fairly conserved (Beyer et al., 2002). The faint immune-reactivity that we observed for the “canonical” alkaline Cor a 9 subunit is most likely due to its homology with the 20.7 kDa IgE-binding subunit. In fact, has to be taken into account that a single amino acid substitution in an allergen can modified its IgE antibody binding capacity or the affinity to the antibody (Crespo et al., 2006). The current MS data provide only a preliminary sequence of the protein, due to the incompleteness of the hazelnut genomic annotation.

The allergenic potential of the alkaline 11S subunit has been already described and assessed for other seeds. Helm et al. 2000a and 2000b identified and characterized a 22 kDa alkaline subunit of glycinin, an 11S globulin-like storage protein, as one of the major soybean (*Glycine max*) allergen (Gly m 6). The 21 kDa alkaline 11S subunit, homologue to soybean G2 glycinin, has been described as the primary allergen of pistachios, cashew nuts (Tawde Pallavi 2004), chickpea. As well as, Ballabio et al., 2010 described a 20 kDa band as the most responsive and recurrently detected peanut polypeptide in 12 allergic children, corresponding to the basic subunit of arachin, the peanut legumin-like globulin. Moreover, the N-terminal sequence was defined the major peptide responsible for cross-reactivity among leguminous seed proteins in peanut allergic children. Recent clinical findings suggest that sensitization to Cor a 9, and less extended to Cor a 11, is age dependent (Verweij et al., 2010) and more in deep may depended on the age of the child. In particular the Cor a 9 is the dominant sensitizing factor in infants (< 1 year) and the additive immunoreactivity to the Cor a 11 increase in older child (7-9 years). IgE-reactivity to Cor a 9 and Cor a 11 may decrease later in life, even when the hazelnut allergy persists. The age-dependent sensitization to Cor a 9 has been assessed using microarrays (Phadia) with commercially available, purified (Cor a 9 and Cor a 11) or recombinant (Cor a 1 and Cor a 8) allergen (Verweij et al., 2010; Verweij et al., 2012). The possible involvement of the 11S isoform in Cor a 9 sensitization cannot be excluded, as the two 11S isoforms are generally co-purified under ordinary separation conditions (Rigby et al., 2008).

[M+H] <sup>+</sup>	De novo sequenced peptides	Top-ranking homologue protein	Notes
978.51	YIQLSAER	identical sequence in gi 224062141 54.7 kDa predicted protein <i>Populus trichocarpa</i>	
1211.5	GVEETFCTLR	GVEETFCT <b>A</b> R in gi 224147821 56.0 kDa Predicted protein Cupin superfamily <i>Populus trichocarpa</i>	<b>G</b> LEETFCTLR in 56.3 kDa 11S globulins of <i>Ficus pumila</i> and GVEETFCT <b>M</b> R of <i>Ricinus communis</i>
1342.6	AGSQGFVWSFK	identical sequence in gi 224126783 55.9 kDa predicted protein <i>Populus trichocarpa</i>	<b>A</b> SS <b>D</b> GFEVWSFK gi 156001070 in 53.2 kDa 11S globulin of <i>Pistacia vera</i>
1386.6	TNDNAQISQLAGR	identical sequence in gi 224147821 56.6 kDa Predicted protein Cupin superfamily <i>Populus trichocarpa</i>	TNDNAQIS <b>P</b> LAGR in gi 75248608 in 59.1 kDa 11S globulin of <i>Corylus avellana</i> TNDNA <b>L</b> INTLSGR in gi 122211016 in 52.7 kDa 11S globulin isoform 4 of <i>Sesamum indicum</i>
1389.8	ALPEDVLLNSYR	ALPED <b>A</b> LLS <b>S</b> TR in gi 34395154 hypothetical 21.9 kDa protein of <i>Oryza sativa</i>	
1400.7	HDGQNLFDWLR	No significant homology	
1453.8	ITSLNSLNPILR	ITSLNSLNPIL <b>K</b> in gi 171853010 53.3 11S allergen of <i>Pistacia vera</i>	

**Table 4.** ESI-Q-TOF MS/MS de novo sequenced tryptic peptides of the 20.7 kDa putative hazelnut allergen and their homology with related 11S globulins from other plants. Amino acidic substitutions with respect to the sequenced peptides are highlighted in bold.

The immunoreactive pattern of hazelnut extracts was compared by immunoblotting with that of the SPT solution by Lofarma used for the clinical diagnosis of food allergies. Proteins extracted from raw hazelnut and the Lofarma solution were electrophoretically separated in presence and absence of  $\beta$ -mercaptoethanol as reducing agent. The Comassie stained 1D SDS-PAGE profile of the SPT solution was quite different from that of the hazelnut extracts of the (Figure 31).



**Figure 31.** CBB R250 SDS-PAGE comparison of PBS proteins from raw hazelnut (1-2) (line 2) and SPT solution (2-3) treated in absence (1-3) and in presence of  $\beta$ -mercaptoethanol (2-4)

This discrepancy can be attributed to the extensive proteolysis of the SPT proteins induced by seed proteases and, to the extraction buffer used and to the presence of stabilizing and denaturing/reducing agents for the conservation. The occurrence of additional immunoreactive small polypeptides (<6 kDa) cannot be ruled out. When immunoassayed with the sera of children allergic to hazelnuts, the ~21 kDa protein was practically the unique IgE-reactive component of both the hazelnut extract and SPT solution (Figure 32).

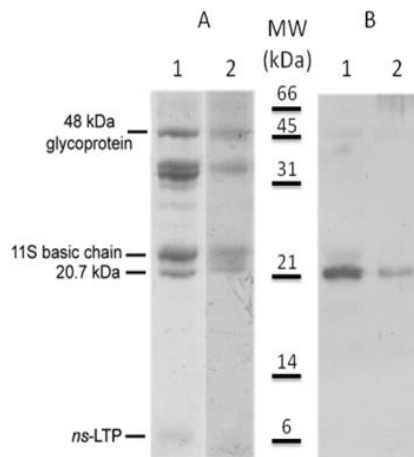


Figure 32. CBB R250 SDS-PAGE 15% (A and B) and immunoblot (1 and 2) stained with a pool of sera from hazelnut allergic children comparison of PBS hazelnut protein extracts (A, 1) and the Lofarma commercial Skin Prick Test solution (B, 2).

To define the pattern cross-reactivity pattern of 1 hazelnut allergic child who showed clinical sensitization to a large number of seed types, among which beans prevailed, the western blotting analysis of extracted proteins from raw and roasted beans flowers was also carried out (Figure 33). A 21 kDa band was again the predominant IgE immunoreactive ones. Similarly, a recent study aimed to define the cross-reactivity among legume seed storage protein in allergic children demonstrate that the basic chain of the 11S globulin like form several legumes (pea, beans, lentils) was the predominant allergic determinant (Ballabio et al., 2010).

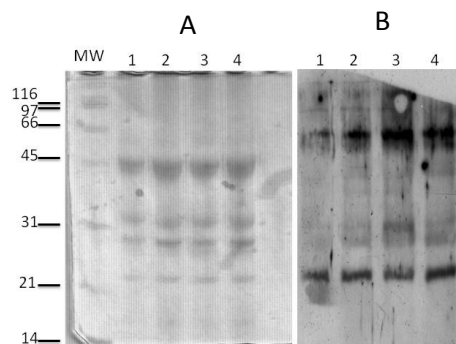


Figure33. (panel a) CBB R250 SDS-PAGE corresponding western blotting stained with sera of bean/hazelnut allergic child, (panel b) crude protein extract from beans defatted flower (line 1-2) and from dried beans flower treated at 80°C for 200 min (line 3-4).

A preliminary proteomic and immunochemical characterization of proteins extracted from roasted Oregon hazelnuts was also carried out. Proteins were extracted with saline buffer and reducing and denaturant buffer. The 1D electrophoretic separation did not highlight substantial modification in terms of extraction buffer at least for the most abundant proteins. When proteins were blotted with a pool of sera from hazelnut allergic children the 20.7 kDa band was still the major IgE binding protein. These results may suggest that the heat treatments do not modified the protein structure enough to completely affect the potential of proteins to elicit allergic reactions.

### 3.5 Conclusion

Sera from 15 hazelnut allergic children recognized as predominant IgE-reactive component a minor ~ 55 kDa protein not previously described. Similar to the hazelnut 11S globulin Cor a 9 allergen, the immunoreactive protein consisted of two subunits linked via a disulfide bridge. Only the 20.7 kDa alkaline subunit exhibited IgE-affinity. The immunogenic subunit was purified by a two-step chromatographic procedure and peptide mass fingerprinting

was unsuccessful in identifying it, due to the incompleteness of the annotated hazelnut genome. Several tryptic peptides were *de novo* sequenced by tandem mass spectrometry and database interrogation showed a high degree of homology with the 11S globulin isoforms of storage proteins from other seeds, some of which have already been reported as food allergens. A clinical SPT screening for validating the actual allergenic potential of the purified 20.7 kDa protein and its larger protein precursor is in progress. The allergenic effects of this new 11S-globulin isoform also should also be studied in allergic individuals from other geographical regions. The protein characterization remains to be completed either at the gene or protein expression levels, for instance, by constructing an opportune probe, based on the sequenced peptides, to clone the corresponding cDNA.

The possible allergenic role of this protein presents a new opportunity for developing molecular assays, more sensitive and specific for diagnostic of hazelnut allergy and the developed of new therapeutic protocols to enhance specific long-term tolerance.

### 3.6 References

- Ballabio C, Magni C, Restani P, Mottini M, Fiocchi A, Tedeschi G, Duranti M. (2010) IgE-mediated cross-reactivity among leguminous seed proteins in peanut allergic children. *Plant Foods Hum Nutr* 65(4):396-402.
- Barre A, Sordet C, Culerrier R, Rancé F, Didier A, Rougé P (2008) Vicilin allergens of peanut and tree nuts (walnut, hazelnut and cashew nut) share structurally related IgE-binding epitopes. *Mol Immunol* 45(5):1231-40.
- Beyer K, Grishina G, Bardina L, Grishin A, Sampson HA (2002) Identification of an 11S globulin as a major hazelnut food allergen in hazelnut-induced systemic reactions. *J Allergy Clin Immunol* 110(3):517-23.
- Crespo JF, James JM, Fernandez-Rodriguez C, Rodriguez J (2006) Food allergy: nuts and tree nuts. *Br J Nutr Suppl* 2:S95-102.
- Flinterman AE, Akkerdaas JH, den Hartog Jager CF, Rigby NM, Fernandez-Rivas M, Hoekstra MO, Bruijnzeel-Koomen CA, Knulst AC, van Ree R, Pasmans SG (2008) Lipid transfer protein-linked hazelnut allergy in children from a non-Mediterranean birch-endemic area. *J Allergy Clin Immunol* 121(2):423-428.
- Garino C, Zuidmeer L, Marsh J, Lovegrove A, Morati M, Versteeg S, Schilte P, Shewry P, Arlorio M, van Ree R (2010) Isolation, cloning and characterization of the 2S albumin, a new allergen from hazelnut. *Mol Nutr Food Res* 54:1257-1265.
- Helm RM, Cockrell G, Connaughton C, Sampson HA, Bannon GA, Beilinson V, Livingstone D, Nielsen NC, Burks AW (2000a) A soybean G2 glycinin allergen. 1. Identification and characterization. *Int Arch Allergy Immunol* 123(3):205-12.
- Helm RM, Cockrell G, Connaughton C, Sampson HA, Bannon GA, Beilinson V, Nielsen NC, Burks AW (2000b) A soybean G2 glycinin allergen. 2. Epitope mapping and three-dimensional modeling. *Int Arch Allergy Immunol* 123(3):213-9.
- Pastorello EA, Vieths S, Pravettoni V, Farioli L, Trambaioli C, Fortunato D, Lüttkopf D, Calamari M, Ansaloni R, Scibilia J, Ballmer-Weber BK, Poulsen LK, Wütrich B, Hansen KS, Robino AM, Ortolani C, Conti A (2002) Identification of hazelnut major allergens in sensitive patients with positive double-blind, placebo-controlled food challenge results. *J Allergy Clin Immunol* 109(3):563-70.
- Rigby NM, Marsh J, Sancho AI, Wellner K, Akkerdaas J, van Ree R, Knulst A, Fernández-Rivas M, Brettlova V, Schilte PP, Summer C, Pumphrey R, Shewry PR, Mills EN (2008) The purification and characterization of allergenic hazelnut seed proteins. *Mol Nutr Food Res* 52 Suppl 2:S251-61.
- Schocker F, Lüttkopf D, Scheurer S, Petersen A, Cisteró-Bahima A, Enrique E, San Miguel-Moncín M, Akkerdaas J, van Ree R, Vieths S, Becker WM (2004) Recombinant lipid transfer protein Cor a 8 from hazelnut: a new tool for in vitro diagnosis of potentially severe hazelnut allergy. *J Allergy Clin Immunol* 113(1):141-7.
- Tawde Pallavi D (2004) Allergenic Cross-Reactivity Between Cashew and Pistachio Nuts. *Electronic Theses, Treatises and Dissertations*, 358.
- Verweij MM, Hagendorens MM, De Knop KJ, Bridts CH, De Clerck LS, Stevens WJ, Ebo DG (2011) Young infants with atopic dermatitis can display sensitization to Cor a 9, an 11S legumin-like seed-storage protein from hazelnut (*Corylus avellana*). *Ebo Pediatr Allergy Immunol* 22(2):196-201.
- Verweij MM, Hagendorens MM, Trashin S, Cucu T, De Meulenaer B, Devreese B, Bridts CH, De Clerck LS, Ebo DG (2012) Age-Dependent Sensitization to the 7S-Vicilin-Like Protein Cor a 11 From Hazelnut (*Corylus avellana*) in a Birch- Endemic Region. *J Investig Allergol Clin Immunol* 22(4): 245-251.



#### **4.**

### **Section 3: Simulated gastrointestinal digestion of hazelnut proteins**

## 4.1 Aim of the study

The aim of the study was to investigate the influence of gastrointestinal digestion on the allergenic potential of crude hazelnut protein extract and on the new 55 kDa isoform of 11S globulin like. Three steps of *in vitro* digestion were followed by electrophoresis and RP-HPLC analysis of the digestion kinetic.

## 4.2. Material

PBS-hazelnut extracted proteins (Mortarella Cv) and the pure 55 kDa IgE binding protein were used. Pepsin, trypsin, chymotrypsin and elastase, carboxypeptidase A, elastase, dithiothreitol (DTT), iodoacetamide,  $\alpha$ -cyano-4-hydroxycinnamic acid and 2,5-dihydroxybenzoic acid were purchased from Sigma (St. Louis, MO, USA). Porcine pepsin, chymotrypsin elastase and trypsin were used assuming that no significant differences in their activity exist with respect to the human counterparts (Hernández-Ledesma et al., 2004; Varilová et al., 2005).

## 4.3 Method

### 4.3.1 Preparation of human BBM

The brush-border membrane (BBM) vesicles from human small intestine were prepared as described by Shirazi-Beechey et al. 1990 with little modification. Frozen surgical specimens of intestinal jejunum (1 cm) were thawed in ice-cold 50 mM mannitol, 2 mM Tris/HCl pH 7.1. Cells were removed from the underlying connective tissue by using a Vibromixer (model E-1, Alpha Laval, UK) at max speed  $2 \times 1$  min. The cell suspension was homogenized at maximum speed with an Ultraturax T 25 (IKA, Works, Inc., USA). 500 mM MgCl<sub>2</sub> stock solution was then added to a final concentration of 10 mM. The suspension was stirred 20 min at 0 °C and then centrifuged at  $3000 \times g$  15 min at 4 °C to eliminate cell debris, basolateral membranes, nuclei, and mitochondria. The supernatant was centrifuged at  $30,000 \times g$  30 min at 4 °C. The pellet was resuspended in 300 mM mannitol, 0.1 mM MgSO<sub>4</sub>, and 2 mM Tris pH 7.4 to give a protein concentration of 10–30 mg/mL and made homogeneous by passing several times through a 27-gauge needle. Shortly before use, the vesicles were thawed on ice and washed with the buffer required for the planned experiments. The purification degree of BBM preparation was determined by measuring sucrase specific activity, as reported below. Sucrase activity was measured using a procedure based on that of Dahlqvist et al., 1968.

### 4.3.2 Gastric–pancreatic and BBM digestion

Hazelnut saline-soluble proteins (200  $\mu$ g) and pure 55 kDa IgE-binding protein (25  $\mu$ g) were dissolved in 5% formic acid and incubated at 37°C with pepsin (1:100 protease to protein, w/w ratio) for 60 min. The incubation period with pepsin mimics the *in vivo* gastric half-emptying time measured by Mah et al., 1994. Before pancreatic digestion, samples were evaporated and washed twice with deionised water. Trypsin (1:100, w/w), chymotrypsin (1:100, w/w), elastase (1:500, w/w) and carboxypeptidase (1:100, w/w) were added in phosphate buffer (pH 7.0) and the mixtures were incubated at 37 °C for 1 h. The reaction was stopped by heating for 5 min and samples were freeze dried. Samples from gastric–pancreatic digestion were dissolved in phosphate buffer pH 7.2 and incubated after BBM supplementation (650 mU/mg) for 1 h.

### 4.3.3 Dot-blot analysis

Peptides were redissolved in 5  $\mu$ L of PBS buffer and spotted on activated PVDF membrane for dot-blotting analysis. Dot blot analysis was performed as previously described (see section 3.3.1).

### 4.3.4 RP-HPLC analysis

Liquid chromatography was performed using a 2.0mm i.d. $\times$ 250 mm, C18, 5mm reverse-phase column (Phenomenex, Torrance, CA, USA) with a flow rate of 0.2 mL/min on a HP1100 modular system (Agilent, Palo Alto, CA, USA). Solvent A was 0.03% trifluoroacetic acid (TFA) (v/v) in water; solvent B was 0.02% TFA (v/v) in acetonitrile. The column was equilibrated at 5% solvent B. Separation of the peptides was effected with a gradient of 5–70% solvent B over 90 min. The column effluent was monitored by UV detection (220 nm) and each peak was manually collected. For HPLC with positive ionization mass spectrometry (LC–ESI/MS) the effluent from the column was injected on-line.

## 4.4 Results and discussion

A simple model for simulated gastrointestinal digestion was applied to hazelnut crude protein extract and the pure ~ 55 kDa IgE binding protein in order to assess the stability of food allergens during digestion and identify the major IgE-binding epitope(s).

The peptides formed in digested fraction were characterized by means of LC/MS. The peptides purified by RP-HPLC were assessed, together with the intact protein, by dot-blot analysis with pool of sera of allergic patients, allowing the estimation of their potential allergenicity and the definition of the epitope(s). The resistance to digestion of proteases could be a major factor for the development of allergic reactions. The proteolytic breakdown of proteins was monitored by RP-HPLC (Figure 34) and SDS-PAGE (Figure 35) after each step of digestion. The most abundant allergens 11S globulin like protein and the 48 kDa glycoprotein, that were clearly evident in the gel of crude extract as large band of approximately 35 (acidic chain) and 25 (basic chain) kDa and 45 kDa, respectively, undergo to a drastic breakdown already during the gastric phase for the combined action of the acid pH and the pepsinolysis. This is demonstrated by the rapid generation of low molecular weight peptides (< 10 kDa) within 1 hour of gastric digestion.

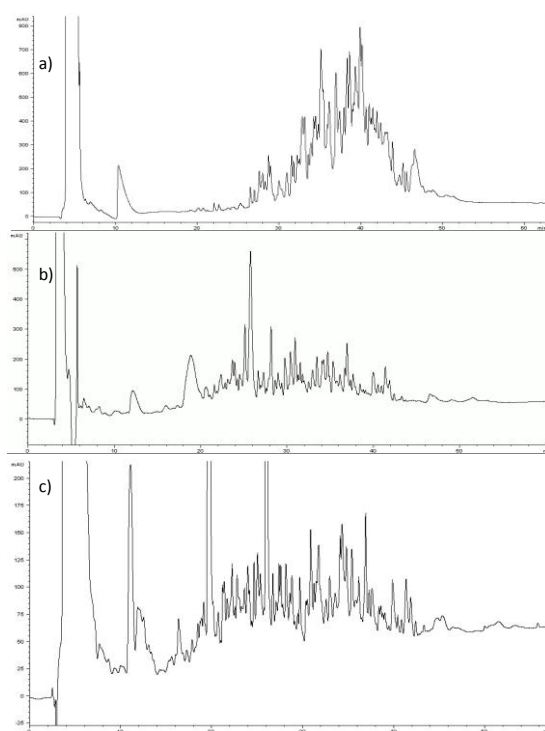


Figure 34. Kinetics of sequential release peptide fragments from crude proteins extract (Cv Mortarella) digested by pepsin (panel a), pool of pancreatic proteases (panel b) and BBM (panel c) monitored by C18 RP HPLC analysis.

Despite the low stability of allergens in the stomach, the total peptide digest of the crude proteins extract and the pure 55 kDa IgE binding protein retained IgE-reactivity when assayed by dot-blot analysis incubated with a pool of sera from 15 hazelnut-allergic children (Figure 36). When the RP-HPLC isolated peptides were assayed by dot-blot analysis, we were not able to detect specific IgE reactivity for anyone's. These results strongly suggest that immunoreactivity is likely due to traces of the undigested/partially digested protein and corroborates the hypothesis of linear epitopes.

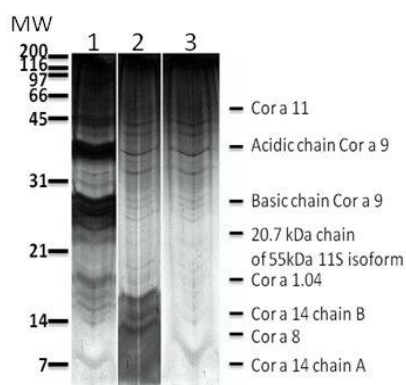


Figure 35. CBB stained 1D SDS-PAGE of PBS extracted proteins from raw hazelnut (Cv Mortarella) (line 1), proteins digested by pepsin (line 2), by a pool of pancreatic enzymes (line 3).

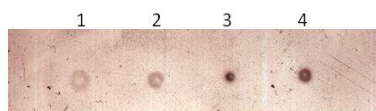


Figure 36. IgE immunodetection of peptides survived to *in vitro* gastrointestinal digestion blotted with a pool of 15 sera from patient allergic to hazelnut, recruited in Southern Italy. Spot 1 - 0.5  $\mu$ g total extracted proteins, Spot 2 - 10.5  $\mu$ g total extracted proteins, Spot 3 - 1  $\mu$ g digesta of total extracted proteins, Spot 4 - 1  $\mu$ g digesta of pure 55 kDa IgE binding protein.

## 4.5 Conclusion

Up to now only few studies about simulated gastrointestinal digestion of hazelnut allergens has been reported in literature. The effect of proteolytic digestion is an important factor for the allergenicity of proteins via the gastrointestinal tract. This stability may be important for both sensitization after crossing through the mucosa and for the eliciting of gastrointestinal symptoms of food allergy.

In this preliminary study we describe a static *in vitro* gastro-duodenal digestion model applied to crude hazelnut extract to predict the susceptibility and the types of transformations during digestion. The resistance of the proteins to proteolysis has been evaluated only by considering its stability in electrophoresis. Our results are in accordance with Vieths et al., 1999, who demonstrated that crude hazelnut extract is extensively susceptible to pepsinolysis. The pool of peptides realized from digestion together with the isolate C18-RP-HPLC peptides were assayed by dot-blot analysis against a pool of 15 sera from allergic sera. Except for the pool of digested peptides from both the total crude extract and the pure 55 kDa IgE binding protein, no IgE immunoreactivity was detected for single peptides. This evidence suggests that probably the immunoreactivity is due to trace of undigested or partially digested protein.

These results suggested moreover that our digestion conditions degraded hazelnut storage protein more extensively compared to the *in vivo* process (Fu 2002). Identification and characterization by nano-LC-MS/MS analysis of peptides realized by digestion are in progress. Further investigations are required to set up appropriate operative conditions to identify IgE-binding domains of putative hazelnut allergen(s) resistant to digestion which would allow to localize the putative epitopes.

## 4.6 References

- Dahlqvist A (1968) Assay of intestinal disaccharidases. *Anal Biochem* 22(1):99-107.
- Fu TJ (2002) Digestion stability as a criterion for protein allergenicity assessment. *Ann N Y Acad Sci* 964:99-110.
- Garcia-Casado G, Sanchez-Monge R, Chrispeels MJ, Armentia A, Salcedo G, Gomez L (1996) Role of complex asparagine-linked glycans in the allergenicity of plant glycoproteins. *Glycobiology* 6(4): 471-477.
- Hernández-Ledesma B, Amigo L, Ramos M, Recio I (2004) Application of high-performance liquid chromatography-tandem mass spectrometry to the identification of biologically active peptides produced by milk fermentation and simulated gastrointestinal digestion. *J Chromatogr A* 1049(1-2):107-14.
- Mah S, Roos N, Benamouzig R, Sick H, Baglieri A, Huneau JF, Tom D (1994) True exogenous and endogenous nitrogen fractions in the human jejunum after ingestion of small amounts of [15N]-labeled casein. *Journal of Nutrition* 124:548-555.
- Shirazi-Beechey SP, Davies AG, Tebbutt K, Dyer J, Ellis A, Taylor CJ, Fairclough P, Beechey RB (1990) Preparation and properties of brush-border membrane vesicles from human small intestine. *Gastroenterology* 98(3):676-85.
- Varilová T, Vráňková A, Pacáková V, Tichá M, Stulík K (2005) Preparation and testing of stationary phases and modified capillaries for affinity chromatography and affinity capillary electrophoresis of pepsin. *J Chromatogr A* 1084(1-2):207-13.
- Vieths S, Reind J, Müller U, Hoffmann A, Hausteiner D (1999) Digestibility of peanut and hazelnut allergens investigated by a simple in vitro procedure. *European Food Research and Technology* 209(6):379-388.

## **Appendix I: Short-Term-Mobility - Infogest COST Action**

## I.1 Introduction

The human gastrointestinal tract is a complex system aimed to the breakdown of both food tissues and components in order to extract the nutrients. During the transit through the digestive system, the food is subjected to drastic changes of pH, that may fall to 2 in the stomach and is finally neutralized in the small intestine, where is mixed with enzymes (amylases, proteases and lipases) and detergents (bile and phospholipids) (Wickham et al., 2009). Several studies have been carried out aimed to the development and the optimization of an *in vitro* digestion model of whole food matrix which may closely mimic the condition and the process that actually occur *in vivo*. The optimal *in vitro* digestion models should be highly reproducible, and offer the advantage of rapid sampling at any time point.

The main stages to be considered in the development of the model are: (i) processing in the mouth, where food is chopped in small pieces, mixed with saliva and partly hydrolyzed by the salivary amylase; (ii) processing in the stomach, where more chemical and mechanical digestion of proteins occurs; (iii) processing in the duodenum.

The static model does not mimic the physical processes that occur *in vivo* (e.g. shear, mixing, hydration, and changing conditions over time) but keep the full range of physical surfactants found in the gastric and duodenal compartments. These systems are far from those likely to be found in the stomach. For instance do not take in account the gradual reduction of pH, stimulated by food arriving in the stomach, which is responsible for the conversion of pepsinogen in pepsin. The impact of the multi-phase nature of digestive system on protein digestibility is taken in account by the inclusion in the model of both the emulsion and liposome within the gastric environment and liposome or micelle phases in duodenal environment. In fact, if on one hand bile acids are potent “digestive surfactants” that promote absorption of lipids, on the other hand can dramatically affect the rate of protein hydrolysis by trypsin and chymotrypsin adsorbing it to oil/water interface. In particulate plant food allergens have the ability to bind lipids and associate with membranes (Wickham et al., 2009).

During my stay to the Institute of Food Research, within the INFOGEST Cost action, has been developed and optimized a static model for the *in vitro* gastrointestinal digestion of complex food matrices. Pasta has been chosen as simple food model to experiment the efficiency of the digestion protocol. The parameters of the *in vitro* enzymatic digestion protocol were selected to mimic *in vivo* hydrolysis of food in terms of biochemical, physical and mechanical conditions: enzyme concentration, pH adjustment (3, then 7), body temperature (37 °C), shaking and adequate incubation time.

## I.2 Material

Spaghetti (Italian brand Napolina for exportation) samples were purchased from a local supplier (100% durum wheat semolina – 12% proteins, 77% carbohydrate, 0.7% lipids). 50g of spaghetti were cooked in 1.5 L water (tap water) added to 3.5g NaCl for 10 min (Hoebler et al., 1998). The weight of the pasta increased by about 250% after cooking. After cooking, pasta was chopped by a mincer.

Simulated saliva fluid (SSF), consisted in 12.53 mM KCl, 0.82 mM KSCN, 19.98 mM KH<sub>2</sub>PO<sub>4</sub>, 7.99 mM NaHCO<sub>3</sub>, 4.10 mM NaCl, 7.49 mM Urea, 0.29 mM MgCl<sub>2</sub>(H<sub>2</sub>O)<sub>6</sub>, 0.003 mM CaCl<sub>2</sub>(H<sub>2</sub>O)<sub>2</sub>.

Simulate gastric fluid (SGF) consisted in 35.09 mM KCl, 0.89 mM KH<sub>2</sub>PO<sub>4</sub>, 12.99 mM NaHCO<sub>3</sub>, 41.06 mM NaCl, 0.22 mM Urea, 0.59 mM MgCl<sub>2</sub>(H<sub>2</sub>O)<sub>6</sub>, 0.001 mM CaCl<sub>2</sub>(H<sub>2</sub>O)<sub>2</sub>, 1.02 mM NH<sub>4</sub>Cl, 0.09 mM Glucuronic acid, 1.86 mM Glucosamine, 1.78 mM Galactose.

Simulated duodenal fluid (SDF) consisted in 6.76 mM KCl, 0.79 mM KH<sub>2</sub>PO<sub>4</sub>, 84.99 mM NaHCO<sub>3</sub>, 32.85 mM NaCl, 1.79 mM Urea, 0.32 mM MgCl<sub>2</sub>(H<sub>2</sub>O)<sub>6</sub>, 0.001 mM CaCl<sub>2</sub>(H<sub>2</sub>O)<sub>2</sub>.

Electrolytic composition of SSF, SGF and SDF was prepared according to Kopf-Bolan et al., 2012.

Enzymes were purchased from Sigma (St. Louis, MO, USA). L- $\alpha$ -Phosphatidylcholine from egg yolk (PC, lecithin grade 1, 99% purity) was obtained from Lipid Products (South Nutfield, Surrey, UK).

## I.3 Methods

### I.3.1 *In vitro* digestion model

Oral phase: 5g of chopped pasta was mixed with 5 mL of simulated saliva fluid (SSF) (50:50, w:v) added with 150 IU corresponding to 50.45 SigmaU of human salivary amylase per mL of SSF.

Gastric phase: The oral digesta was mixed 50:50 (w:v) with SGF, the pH was rapidly decrease to 3 by adding HCl and the suspension was added to 0.17 mM final concentration of gastric liposome (egg lecithin prepared in a vesicular form, Moreno et al., 2005), 1000 U of porcine pepsin per mL of SGF and 80U of fungal lipase per mL of SGF. The pH of the digesta was adjusted to 3 and the incubation was carried out for 2 hours in shaking incubator 37° C /170 rpm. Fractions (250  $\mu$ L) were collected at different times (0, 30, 60, 90 and 120 min) and the proteolysis was monitored by SDS-PAGE analysis.

Duodenal phase: gastric digesta (50:50, v:v) was added to simulated duodenal fluid (SDF) containing 10 mM bile salts (5 mM sodium taurocholate - 5 mM sodium glycodeoxycholate), 1.8 mM (final concentration) egg lecithin and pancreatin (. The amount of pancreatin was calculated on the base of the measured activity of trypsin (corresponded

to 7.22 TAME units trypsin/mg pancreatin) to have 100 U of trypsin/mL final volume. Fractions (250  $\mu$ L) were collected at different times (0, 5, 15, 30, 60, 90, 120 min) the digestion was monitored by SDS-PAGE analysis.

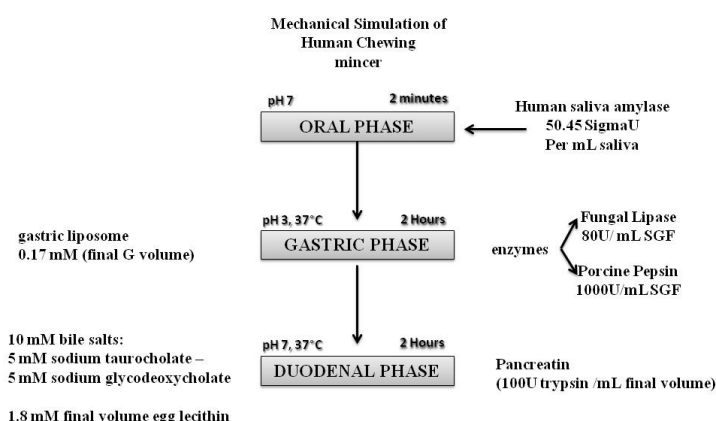


Figure 37. Schematic representation of the optimized *in vitro* digestion model.

### I.3.2 SDS-PAGE analysis

SDS-PAGE was performed (see section 2.4.2.2) on 4–12% gradient gels run for 35 min at 200 V in MES buffer. Fractions were centrifuged and 65  $\mu$ L of supernatant were added to 10  $\mu$ L of 0.5 mM DTT and 25  $\mu$ L of NuPAGE LDS sample buffer (4X). Samples were heated for 10 minutes at 70 °C and 20  $\mu$ L for each gastric and 10  $\mu$ L for each duodenal fraction respectively have been loaded into adjacent wells in the gel.

Samples of cooked pasta and pancreatin separately were incubated for 60 minutes at room temperature with 1 mL of extraction solution (250  $\mu$ L Nu PAGE LDS sample buffer + 650  $\mu$ L water + 100  $\mu$ L DTT (0.5M, 77.5 mg/mL)). After 10 minutes of heating at 70 °C, the solution was centrifuged and the supernatant was collected for SDS-PAGE analysis. The amount of pancreatin loaded into the gel respects the concentration of pancreatin in the final digesta (13.85 mg/mL). Gels were fixed with 50 mL of sulphosalicylic acid/TCA, 10.5/36, in 300 mL of water for 30 minutes, washed twice with water and stained using colloidal Coomassie G250 (Invitrogen).

### I.4 Results

Gastric digestion fractions of cooked pasta were monitored by SDS-PAGE as showed in Fig 38 (panel a). As expected, the intensity of bands at low molecular weight increased with the evolution of digestion from 0 to 120min (lines 4-8). The main band at nearly 35kDa corresponded to pepsin enzyme. High molecular weight protein observed in untreated pasta sample (lines 1 and 2, panel a), were absent in digested samples (lines 4-8, panel a). A probably explanation was because gliadin and glutenin were not soluble in SSF.

The gastric digesta showed the presence of undigested starch particles which are quickly hydrolysed within the first 30 minutes of duodenal digestion.

The intensity and number of bands dramatically increased after duodenal digestion, even if the high concentration of pancreatin (line 1, panel b) masks the wheat proteins profile. A large number of proteins seem to be resistant to enzyme degradation although the concentration of peptides lower than 7.5 kda increased at longer incubation times (lines 3-9, panel b).

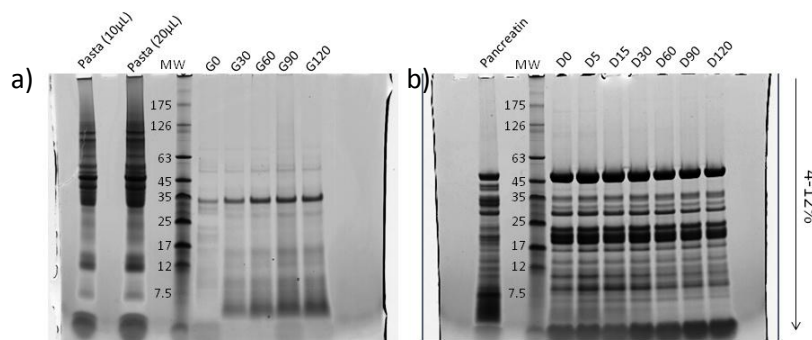


Figure 38. Kinetic of pasta digestion (panel a) SDS-PAGE (4-12%) Coomassie stained - fractions collected from gastric digesta at time points: 0, 30, 60, 90 and 120 min; (panel b) SDS-PAGE (4-12%) Coomassie stained - fractions collected from duodenal digesta at time points: 0, 5, 15, 30, 60, 90 and 120 min.



## **I.5 References**

Hoebler C, Karinthe A, Devaux MF, Guillon F, Gallant DJ, Bouchet B, Melegari C, Barry JL (1998) Physical and chemical transformations of cereal food during oral digestion in human subjects. *Br J Nutr* 80(5):429-36.

Moreno FJ, Mackie AR, Mills ENC (2005) Phospholipid interactions protect the milk allergen alpha-lactalbumin from proteolysis during in vitro digestion. *J Agric Food Chem* 53:9810-9816.

Kopf-Bolanz KA, Schwander F, Gijs M, Vergères G, Portmann R, Egger L (2012) Validation of an in vitro digestive system for studying macronutrient decomposition in humans. *J Nutr* 142(2):245-50.

Wickham M, Faulks R, Mills C (2009) In vitro digestion methods for assessing the effect of food structure on allergen breakdown. *Mol Nutr Food Res* 53(8):952-8.

## **Appendix II: Scientific production**

## Papers:

- Nitride C, Mamone G, Picariello G, Mills C, Nocerino R, Berni Canani R, Ferranti P The basic subunit of a new 11S globulin isoform is a putative hazelnut (*Corylus avellana*) allergen. *Submitted*.
- Amigo-Benavent M, Nitride C, Bravo L, Ferranti P, del Castillo MD Stability and bioactivity of a Bowman–Birk inhibitor in orange juice during processing and storage. *Food Funct.*, 2013, Accepted 05 Mar 2013, cDOI: 10.1039/c3fo30354c.

## Food & Function

RSC Publishing

PAPER

View Article Online  
View Journal

### Stability and bioactivity of a Bowman–Birk inhibitor in orange juice during processing and storage

Cite this: DOI: 10.1039/c3fo30354c

Miryam Amigo-Benavent,<sup>a</sup> Chiara Nitride,<sup>b</sup> Laura Bravo,<sup>a</sup> Pasquale Ferranti<sup>b</sup> and M. Dolores del Castillo<sup>a,c</sup>

The present research aimed to evaluate the feasibility of a soybean Bowman–Birk inhibitor (BBI) as a natural functional food ingredient. The influence of food processing, storage and digestion on the structure and bioactivity of BBI added to a food matrix was evaluated. The cytotoxic effect of the digested samples was also tested with the aim to provide information regarding the safe use of BBI as a functional food ingredient. Samples of freshly squeezed orange juice (OJ) and two orange juice model systems (OJ-ms) supplemented and non-supplemented with BBI were prepared and processed mimicking pasteurization and sterilization industrial conditions. Moreover, pasteurized samples were stored at 4 °C for two months. The samples were digested *in vitro* under oral-gastrointestinal physiological conditions. Results seem to indicate that the activity of BBI supplemented to the food matrix sufficiently survives thermal processing, storage and digestion processes. Changes in BBI bioactivity may be ascribed among others to the Maillard reaction. Formation of early Maillard reaction products also called Amadori products has been detected in the food sample. No cytotoxic effect was observed for the samples under study. In conclusion, our findings support that BBI has significant potential as a natural functional food ingredient in pasteurized orange juice stored at 4 °C.

Received 30th November 2012

Accepted 5th March 2013

DOI: 10.1039/c3fo30354c

www.rsc.org/foodfunction

- Picariello, G., Mamone, G., Nitride, C., Addeo, F., Camarca, A., Vocca, I., Gianfrani, C., Ferranti, P. 2012. Shotgun proteome analysis of beer and the immunogenic potential of beer polypeptides. *J Proteomics*. Jul 31; 75(18):5872–5882.

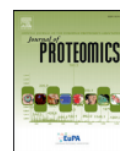
JOURNAL OF PROTEOMICS 75 (2012) 5872–5882



Available online at www.sciencedirect.com

SciVerse ScienceDirect

www.elsevier.com/locate/jprot



### Shotgun proteome analysis of beer and the immunogenic potential of beer polypeptides

Gianluca Picariello<sup>a,b,\*</sup>, Gianfranco Mamone<sup>a</sup>, Chiara Nitride<sup>a,b</sup>, Francesco Addeo<sup>a,b</sup>, Alessandra Camarca<sup>a</sup>, Immacolata Vocca<sup>a</sup>, Carmen Gianfrani<sup>a,c</sup>, Pasquale Ferranti<sup>a,b</sup>

<sup>a</sup>Istituto di Scienze dell'Alimentazione (ISA) — CNR, Via Roma 64, 83100 Avellino, Italy

<sup>b</sup>Dipartimento di Scienza degli Alimenti, University of Naples "Federico II," Parco Gussone, Portici (NA) 80055, Italy

<sup>c</sup>European Laboratory for the Investigation of Food Induced Diseases (ELFID), University of Naples "Federico II," Naples, Italy

#### ARTICLE INFO

Article history:  
Received 26 June 2012  
Accepted 22 July 2012  
Available online 31 July 2012

Keywords:  
Beer  
Shotgun proteomics  
Hordeins  
Gluten-like epitopes  
Celiac disease  
Allergens

#### ABSTRACT

The majority of beer proteins originate from barley (*Hordeum vulgare*) which is used for brewing. Barley is known to contain celiacogenic gliadin-like prolamins (hordeins) along with other immunogenic proteins which endure malt proteases and the harsh conditions of brewing. In addition, a multitude of peptides that may retain or even amplify the immune-stimulating potential is released in beer because of proteolysis.

The comprehensive annotation of the beer proteome is challenged both by the high concentration range of the protein entities and by a severe degree of processing-induced modifications. Overcoming the pitfalls of the classical two-dimensional electrophoresis approach coupled to mass spectrometry (MS), the gel-free shotgun proteomic analysis expanded the current inventory of a popular Italian beer to 33 gene products, including traces of intact B- and D-hordeins and 10 proteins from *Saccharomyces* spp.

The high performance liquid chromatography–electrospray MS/MS peptidomic analysis of the low-molecular weight beer components disclosed a panel of hordein-derived peptides that encrypt gluten-like sequence motifs, potentially harmful to celiacs. The presence of anti-gliadin IgA-immunoresponsive prolamins was assayed by Western and dot blot using sera of N=4 celiac patients. Gliadin-reactive T-cell lines isolated from the intestine of N=5 celiacs activated an IFN-γ response when challenged with deamidated beer polypeptides.

© 2012 Elsevier B.V. All rights reserved.

- Gallo, P., Nitride, C., Nasi, A., Gualtieri, L., Ferranti, P. 2012. Monitoring of algal biotoxins in sea products and dietary supplements: development of an analytical method to ensure food quality and safety. *Ingredienti alimentari*. Chirioti editore Oct 64(11):6–11 (see abstract below).

Abstract: Cyanobacteria (*Cyanophyta* or blue-green algae), are gram negative and photoautotrophs eubacteria. They produce several different biotoxins (cytotoxins, epatoxins, and neurotoxins) that can represent a potential hazard for human health. Among these, Microcystins (MCs) are a group of cyclic heptapeptides showing potent hepatotoxicity and activity as tumour promoters. The occurrence of microcystin-containing blooms in freshwaters has been implicated in several animal and human poisoning outbreaks worldwide. For this reason the analysis of MCs is of growing interest for water surveillance authorities, who need to evaluate analytical strategies for monitoring and characterizing of seaweed blooms on the base of the biotoxins produced. The characterization of the biotoxins produced by several blooms of *Planktothrix rubescens* and other toxic algae in several Italian lakes such as Lake Occhito near Foggia and Lake Averno, near Naples, was obtained using mass spectrometry. This strategy allowed to obtain the characterization and the quantitative evaluation of very low amount of biotoxins. The analysis was then extended to the set-up of a method for quantitative determination of MCs in food integrators for humans and animals.

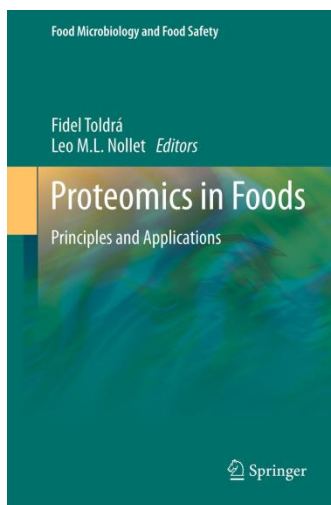
## Conference proceedings:

- De Simone, C., Mamone, G., Nasi, A., Schettino, S., Nitride, C. & Ferranti P. 2010 Proteomic and metabolomic characterization of hazelnut varieties typical of regione Campania. Proc. 5th ItPA Annual National Conference, Florence S5.1
- Nitride, C. and Ferranti, P. 2010 Proteomic, metabolomic, and biological activities of dietary seed protein. proc. 15th Workshop on the “Developments in the Italian PhD Research on Food Science Technology and Biotechnology” pp. 345-346, University of Naples – Federico II, Portici, Italy.
- Nitride, C., Picariello, G., Gualtieri, L., Mamone, G. and Ferranti, P. 2011 Determination and characterization of toxic glycoproteins in bean flour-based products: evaluation of techniques to assure food quality and safety. Proc. 10° Congresso Italiano di Scienza e Tecnologia degli Alimenti (CISETA). Milano (Mi), Italia. In press (see pg. 62)
- Gallo, P., Nitride, C., Nasi, A., Gualtieri, L. and Ferranti, P. 2011 Assessment of biotoxins in fish and dietary supplements: developing of an analytical method to ensure the quality and safety of food. Proc. 10° Congresso Italiano di Scienza e Tecnologia degli Alimenti (CISETA). Milan (Mi), Italy in press.
- Nitride, C., Mamone, G., Picariello, G., Mills, C., Berni Canani, R., Nocerino, R. and Ferranti, P. 2012 Proteomic and immunoassay characterization of a new allergen from hazelnut (*Corylus avellana*). Proc. 7th ItPA Annual National Conference, Viterbo. **Poster won the third prize in the best poster award competition** (see pg. 63)
- Picariello, G., Mamone, G., Nitride, C., Camarca, A., Gianfrani, C. and Ferranti, P. Shotgun proteome analysis of beer and immunogenic potential of beer polypeptides. proc. 7th ItPA Annual National Conference, Viterbo (2012). **Poster won the first prize in the best poster award competition.**
- Nitride, C., Mamone, G., Picariello, G., Mills, C., Berni Canani, R., Nocerino, R. and Ferranti, P. 2012 Identification of a new food allergen from hazelnut (*Corylus avellana*): proteomic and immunoassay based approach. Proc. EuPA 2012 Scientific Congress. New horizons and applications for Proteomics. (see pg. 64).
- Nitride, C., Ferranti, P. 2012 Proteomic and immunoassay characterization of a new food allergen from hazelnut (*Corylus avellana*). Proc. XVII Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, University of Bologna, Cesena, 19-21 September.
- Nitride, C., Mamone, G., Picariello, G., Mills, C., Berni Canani, R., Nocerino, R., Ferranti, P. 2012 Proteomic and immunoassay characterization of a new food allergen from hazelnut (*Corilus avellana*). PO23 / Digestive and Liver Disease 44 Suppl. 4 S259–S292.
- Nitride, C., Mamone, G., Picariello, G., Ferranti, P. Simulated gastrointestinal digestion of hazelnut allergens. The 2nd International Conference on Food Digestion - 6th to 8th March 2013 in Spain, Madrid (see pg. 65)

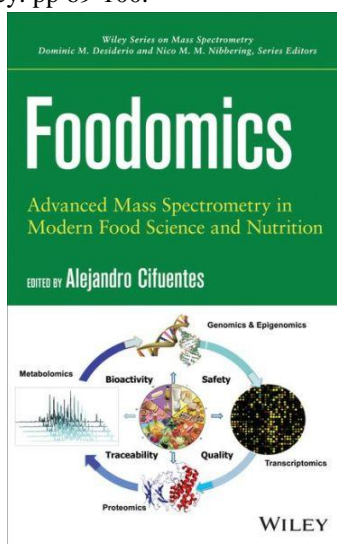
## Book Chapters:

- Picariello, G., Mamone, G., Nitride, C., Addeo, F., Iimure, T. & Ferranti, P. 2013 Beer proteomics. In “Proteomics in food Principles and Applications” (Food Microbiology and Food Safety / Research and Development). Ed L. Nollet and F. Toldrà. Springer 2013. Volume 2, pp 399-424.

- Ferranti, P., Mamone, G., Picariello, G., Nitride, C. & Addeo, F. 2013 The role of proteomics in the discovery of marker proteins of food adulteration. In “Proteomics in food Principles and Applications“ (Food Microbiology and Food Safety/Research and Development). Ed L. Nollet and F. Toldrà. Springer Volume 2, pp 465-501.



- Picariello, G., Mamone, G., Addeo, F., Nitride, C. & Ferranti P. 2013 Proteomic-based techniques for characterization of food allergens. In Foodomics: Advanced Mass Spectrometry in Modern Food Science and Nutrition. Ed. A. Cifuentes –Wiley. pp 69-100.



## Determinazione e caratterizzazione di lectine tossiche in prodotti a base di farina di fagiolo (*Phaseolus vulgaris*): valutazione di tecniche capaci di assicurare la qualità e la sicurezza degli alimenti

Chiara Nitride<sup>a</sup>, Gianluca Picariello<sup>b</sup>, Antonella Nasi<sup>a</sup>, Liberata Gualtieri<sup>a</sup>, Gianfranco Mamone<sup>b</sup>, Pasquale Ferranti<sup>a</sup>

<sup>a</sup> Dipartimento di scienze degli alimenti, Università di Napoli "Federico II", Portici (NA), Italy

<sup>b</sup> Istituto di Scienze dell'Alimentazione - CNR, Avellino, Italy

### INTRODUZIONE

Le fitoemoagglutinine (PHA) sono lectine (proteine in grado di legare specificamente i carboidrati) che rappresentano fino al 12% delle proteine di riserva nei fagioli crudi. Il loro contenuto varia in relazione a fattori genetici ed ambientali, nonché ai trattamenti tecnologici. Benché termolabili, costituiscono un fattore antinutrizionale poiché responsabili di severe gastroenteriti correlate al consumo di fagioli in prodotti industriali (zuppe, minestre, liofilizzati) non correttamente trattati. Un'ulteriore causa di intossicazione può essere legata al consumo di "gluco-block", integratori alimentari utilizzati per curare l'obesità cronica a base di estratti di fagiolo arricchiti in lectine ed inibitori di  $\alpha$ -amilasi.

Il contenuto in lectine attive è determinato con il saggio di emagglutinazione (HA), poco sensibile e aspecifico.

In questo studio è stata sviluppata una metodica di analisi delle PHA estratte da farina di fagiolo per identificare e monitorare modificazioni strutturali indotte dal trattamento termico e un'eventuale correlazione tra struttura (glico)proteica e tossicità. L'applicazione di tecniche di proteomica nell'analisi quali-quantitativa dei campioni contaminati è risultata risolutiva sia per la maggiore sensibilità ed accuratezza con cui ha permesso la quantificazione di lectine attive anche in campioni negativi al saggio di eritroagglutinazione sia perché ha messo in evidenza una possibile modificazione strutturale a carico delle catene oligosaccaridiche potenzialmente coinvolta nell'attività tossica.

### CAMPIONI ANALIZZATI

Sono state analizzate farine di fagiolo destinate alla produzione di piatti pronti liofilizzati quali pasta e fagioli, zuppe e creme, commercializzati in Italia ed in paesi esteri. I campioni in esame hanno subito subito lo stesso processo di trasformazione: macinazione ed essiccazione ad 80 °C per 200 minuti. Sono stati causa di intossicazione alimentare per questo ritirati dal commercio ed analizzati allo scopo di definirne la causa. La presenza delle lectine è stata testata mediante il saggio di eritroagglutinazione ed ha dato risultati contrastanti in alcuni casi esito positivo in altri esito negativo.

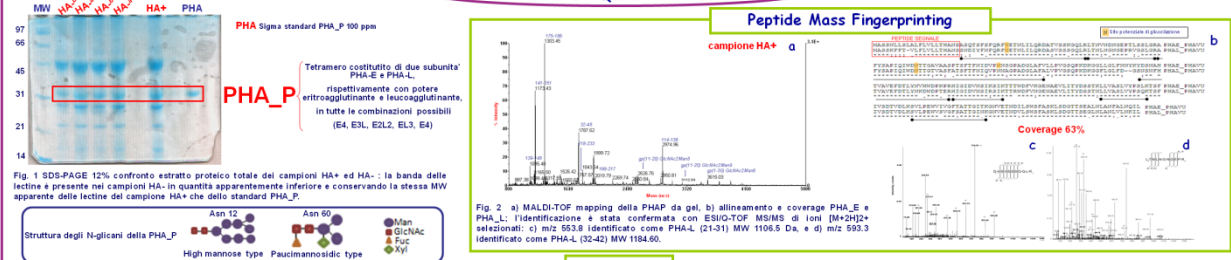
Un campione contaminato positivo al saggio di eritroagglutinazione (HA+)

Due campioni negativi al saggio di eritroagglutinazione. (HA-a, HA-b)

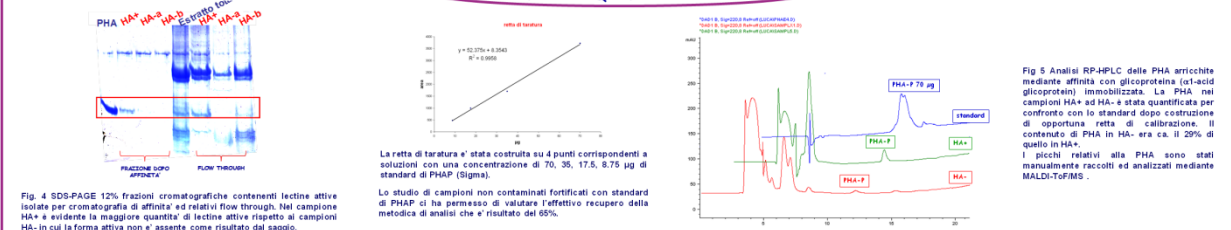
### STRATEGIA ANALITICA



### ANALISI QUALITATIVA



### ANALISI QUANTITATIVA



### CONCLUSIONI

• Sia l'analisi quantitativa che qualitativa ha messo in evidenza la presenza di lectine attive nei campioni negativi al saggio di eritroagglutinazione. L'approccio proteomico si è rivelato molto più accurato e sensibile nella valutazione della presenza della tossina permettendone la quantificazione fino allo 0.75% (w/w) dell'estratto proteico totale.

• L'analisi qualitativa ha evidenziato la ridotta presenza nei campioni sottoposti ad un corretto trattamento termico del glicopeptide "paucimannosic-type" legato ad Asn60 che ci permette di ipotizzare un suo eventuale coinvolgimento nell'azione tossica della lectina. La validazione di questo aspetto renderebbe il glicano paucimannosidico un marker del processo di inattivazione potenziale target di saggi tipo ELISA per quantificare, in modo altamente specifico ed affidabile le lectine attive eventualmente presenti nei prodotti processati.





# Proteomic and immunoassay characterization of a new allergen from hazelnut (*Corylus avellana*)



Chiara Nitride<sup>a,b</sup>, Gianfranco Mamone<sup>b</sup>, Gianluca Picariello<sup>b</sup>, Clare Mills<sup>c</sup>, Roberto Berni Canani<sup>d</sup>, Rita Nocerino<sup>d</sup>, Pasquale Ferranti<sup>a,b</sup>.

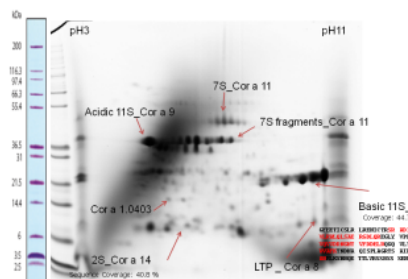
<sup>a</sup>Dipartimento di Scienza degli Alimenti, University of Naples 'Federico II', Parco Gussone, Portici (NA) 80055, Italy; <sup>b</sup> Istituto di Scienza dell'Alimentazione (ISA), CNR, Via Roma 54, 83100 Avellino, Italy; <sup>c</sup> Institute of Food Research, Norwich, UK; <sup>d</sup> Department of Pediatrics and European Laboratory for the Investigation of Food Induced Diseases, University of Naples 'Federico II', Italy.

Corresponding author: Chiara Nitride, PhD student E-mail address: chiara.nitride@unina.it

## Introduction

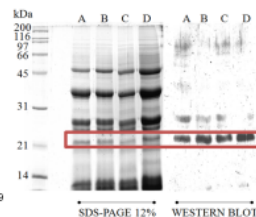
Hazelnut (*Corylus avellana*) is one of the most common causes of lifetime lasting IgE-mediated food allergy. Immune reactions to hazelnut range from mild oral allergy syndromes to severe life-threatening anaphylaxis. The immune reaction in predisposed individuals can be triggered by at least five protein fractions (11S globulin, 7S glycoprotein, 2S albumin, ns-LTP, Cor a1) [1]. The predominance of specific allergens appears to be associated to the geographical origin of allergic subjects. The complexity of the hazelnut allergen pattern challenges a clear definition of both the allergens and the allergenic determinants (epitopes). Therefore, it is still missing for hazelnut a program of allergen standardization in order to support innovative diagnostic and therapeutic approaches. The aim of our investigation is to identify allergenic determinants in pediatric subjects from Campania region (Italy) diagnosed with hazelnut allergy

## 2D-PAGE – Peptide Mass Fingerprinting

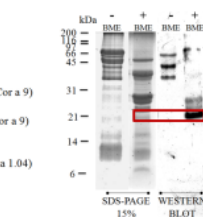


2D IEF/SDS-PAGE of proteins from Cv Mortarella (Coomassie staining). Major protein spots were identified by MALDI-TOF MS-based Peptide Mass Fingerprinting (PMF).

## Immunochemical assay

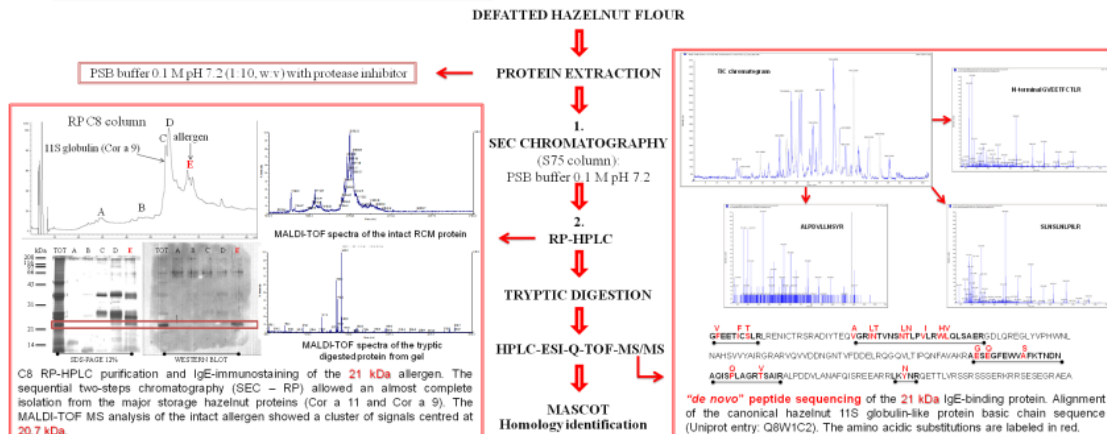


IgE Western blot of hazelnut proteins with 4 allergic children's sera shows immunoreactivity exclusively to a 21 kDa band. Proteins were extracted from three hazelnut autochthonous cultivars from Campania region (A. Tonda, B. San Giovanni, C. Mortarella) and one from Oregon (D). MS/MS analysis and data base searching of the 21 kDa target protein did not match any known hazelnut protein.



Western blot of hazelnut proteins separated without (-BME) and with β-mercaptoethanol (+BME). Three bands in the 35-60 kDa range and only one of 21 kDa were IgE reactive in the -BME and +BME conditions, respectively. The IgE-binding protein is constituted by two or more disulfide-linked subunits, indicative of a 11S-like protein.

## Purification and partial amino acid sequence analysis



## Conclusions

A 21 kDa hazelnut protein subunit, not annotated so far, was the unique IgE-binding protein for 4/4 sera of allergic children from Campania region. The allergen is expressed in all investigated hazelnut cultivars.

The SDS\_PAGE under reducing and non reducing condition shows that the allergen shares structural traits with the hazelnut 11S globulin-like proteins (Cor a 9) [2], being composed by two or more protein subunits, linked via a disulphide bond. Exclusively the monomeric 21 kDa subunit exhibited antigenic properties.

The mass spectrometry-based characterization, also including the *de novo* sequencing of tryptic peptides, provided evidence of the high homology degree between the unknown IgE-binding polypeptide with the 11S globulin-like storage proteins that are expressed in several other seeds: 11S globulin isoform 1B *Ficus pumila* (Uniprot entry A1E0V5), v 2.0101 allergen 11S globulin precursor *Pistacia vera* (Uniprot entry: B7P073), 11S globulin isoform 4 *Sesamum indicum* (Uniprot entry Q2XSW6), predicted protein Cupin superfamily *Populus trichocarpa* (NCBI entry: gi|224126783).

[1] Pastorello EA, Vieths S, Pravettoni V, Farioli L, Trambaioli C, Fortunato D, Lüttkopf D, Calamari M, Ansaloni R, Schibila J, Balfmeier-Viehe BK, Poulsen LK, Wüthrich B, Hansen KS, Robino AM, Citralani C, Conti A. Identification of hazelnut major allergens in sensitive patients with positive double-blind, placebo-controlled food challenge results. *J Allergy Clin Immunol*. 2002;109(3):563-70.  
[2] Beyer K, Grishina G, Bardina L, Grishin A, Sampson HA. Identification of an 11S globulin as a major hazelnut food allergen in hazelnut induced systemic reactions. *J Allergy and Clin Immunol*. 2002;110(3):517-525

# Identification of a new food allergen from hazelnut (*Corylus avellana*): proteomic and immunoassay based approach

Chiara Nitride<sup>a,b</sup>, Gianfranco Mamone<sup>b</sup>, Gianluca Picariello<sup>b</sup>, Clare Mills<sup>c</sup>, Roberto Berni Canani<sup>d</sup>, Rita Nocerino<sup>d</sup>, Pasquale Ferranti<sup>a,b</sup>

<sup>a</sup>Dipartimento di Scienze degli Alimenti, University of Naples "Federico II", Parco Gussone, Portici (NA) 80055, Italy; <sup>b</sup>Istituto di Scienze dell'Alimentazione (ISA), CNR, Via Roma 64, 83100 Avellino, Italy; <sup>c</sup>Institute of Food Research, Norwich, UK; <sup>d</sup>Department of Pediatrics and European Laboratory for the Investigation of Food Induced Diseases, University of Naples "Federico II", Italy

Corresponding author: Chiara Nitride, PhD student E-mail address: chiara.nitride@unina.it

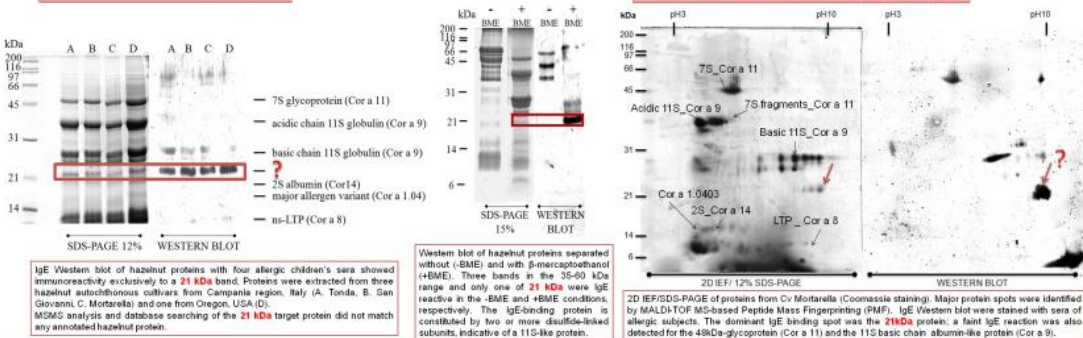
## Introduction

Hazelnut (*Corylus avellana*) is one of the most common lifetime-lasting IgE-mediated cause of food allergy. Immune reactions to hazelnut range from mild oral allergy syndromes, which are caused by cross-reactivity with tree pollen, to severe life-threatening anaphylaxis. Hazelnut allergy is more frequent in infancy than in adulthood and its prevalence varies among countries (0.4-1%).

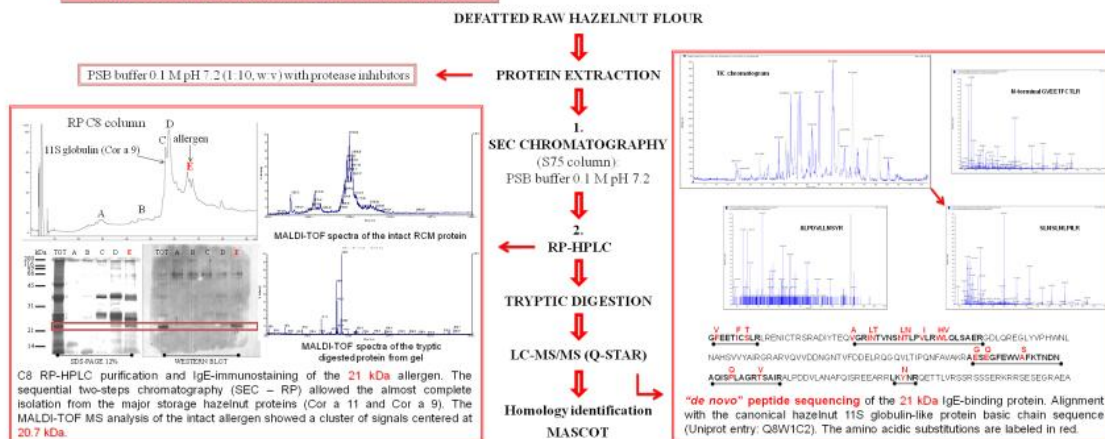
Over the years different studies aimed to identify hazelnut allergenic determinant(s) have been carried out. To date several hazelnut allergenic proteins have been identified and characterized. The predominance of specific allergens appears strictly related to the geographical origin of the patients [1]. Cor a 1.04, related to the birch aeroallergen Bet v 1 (from *Betula verrucosa*), is considered the major allergen in Northern Europe [3]. 11S globulin (Cor a 9), consisting of a 30-40 kDa acid and a 20-25 kDa basic subunits linked via an inter-chain disulfide bridge, is the principal non-pollen related allergen in United States [2]; non-specific-lipid transfer protein (ns-LTP), referred to as a Cor a 8, is supposed to be the predominant allergen in the Mediterranean area [1]. Additional storage proteins such as the 48 kDa-glycoprotein (Cor a 11) and the 2S albumin (Cor a 14) are also reported as potential food allergens.

In this study we investigated the IgE-immune-affinity of hazelnut proteins using sera of hazelnut allergic children from Southern Italy (n=4). Proteomic strategies were applied to the identification and the characterization of the allergenic determinant(s).

## Immunochemical assays



## Purification and partial amino acid sequence analysis



## Conclusions

Western blot with IgE-immunostaining showed that all sera (4/4 sera of allergic children from Southern Italy) recognized a not yet described protein of about 55 kDa consisting of at least two subunits bound via disulfides. Under reductive conditions, a 21 kDa subunit was the unique immunoreactive band. The allergen is expressed in all investigated hazelnut cultivars.

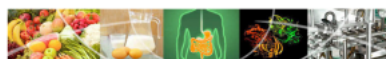
The mass spectrometry-based characterization, also including the *de novo* sequencing of tryptic peptides, provided evidence of the high homology degree between the unknown IgE-binding polypeptide with the 11S globulin-like storage proteins that are expressed in several other seeds. 11S globulin isoform 1B *Ficus pumila* (Uniprot entry A1E0V5), v 2.0101 allergen 11S globulin precursor *Pistacia vera* (Uniprot entry B7P073), 11S globulin isoform 4 *Sesamum indicum* (Uniprot entry Q2XSW6), predicted protein Cupin superfamily *Populus trichocarpa* (NCBI entry: gij224126783).

The IgE binding properties were retained by the pool of peptides that survived a simulated in vitro gastrointestinal digestion, thus suggesting that antigenic determinants are linear epitopes, consistently with the clinical manifestations of the allergic events.

These results open a new scenario for planning innovative diagnostic and therapeutic intervention approaches.

[1] Pastorelli EA, Vietti S, Pravattoni V, Farioli L, Tramballi C, Fortunato D, Lüttkopf D, Calamari M, Ansaloni R, Schölla J, Ballmer-Weber BK, Poulsen LK, Wüthrich B, Hansen KS, Robino AM, Ortolani C, Conti A. Identification of hazelnut major allergens in sensitive patients with positive double-blind, placebo-controlled food challenge results. *J Allergy Clin Immunol*. 2002;109(3):563-70.  
[2] Beyer K, Grisham G, Bardini A, Sampson HA. Identification of an 11S globulin as a major hazelnut food allergen in hazelnut induced systemic reactions. *J Allergy and Clin Immunol*. 2002;110(3):517-523.  
[3] Lüttkopf D et al. Comparison of four variants of a major allergen in hazelnut (*Corylus avellana*) Cor a 1.04 with the major hazelnut allergen Cor a 1.01. *Mol Immunol*. 2002;38(7):515-25, Jan.





INFOGEST  
International Conference  
Madrid, Spain  
6-8 March 2013



# Simulated gastrointestinal digestion of hazelnut allergens

C. Nitride<sup>1,2\*</sup>, G. Mamone<sup>1</sup>, G. Picariello<sup>1</sup>, P. Ferranti<sup>1,2</sup>

<sup>1</sup>Department of Food Science, University of Naples "Federico II", Italy; <sup>2</sup> Institute of Food Science (ISA), CNR, Italy;

Corresponding author: Chiara Nitride, PhD student E-mail address: chiara.nitride@unina.it

## Introduction

Hazelnut (*Corylus avellana*) ranks among the major sources of food allergens. Hazelnut allergy is more frequent in infancy than in adulthood and its prevalence exhibits geographical variation. Symptoms of allergy to hazelnut range from mild oral allergy syndromes, which are caused by cross-reactivity with tree pollen, to severe life-threatening anaphylaxis. In a previous work we have identified and partly characterized the alkaline 21 kDa subunit of a-55 kDa 11S globulin-like protein isoform as the predominant IgE-binding protein for 15/15 hazelnut allergic children recruited from Southern Italy. Food-derived "type-I" linear epitopes have been claimed to be digestion stable. Thus, with the aim of identifying the major IgE-binding epitopes, we simulated the *in vitro* gastrointestinal digestion of both the crude PBS-soluble fraction of raw hazelnut and the HPLC-purified-55 kDa putative allergen.



## Analytical Approach

### Purification of 55 kDa 11S globulin-like protein isoform

DEFATTED RAW HAZELNUT FLOUR

↓  
**PROTEIN EXTRACTION**

PSB buffer 0.1 M pH 7.2 (1:10, w:v) with protease inhibitor

↓  
**SEC CHROMATOGRAPHY**

(S75 column):  
PSB buffer 0.1 M pH 7.2

↓  
**2. RP-HPLC**

### *In vitro* gastrointestinal digestion model of proteins

CRUDE EXTRACT/PURE PROTEINS

↓  
**GASTRIC DIGESTION**

Pepsin (1:100 protease to protein, w/w ratio) 5% formic acid, 37 °C for 1 h.

↓  
**DUODENAL DIGESTION**

Trypsin (1:100, w/w), chymotrypsin (1:100, w/w), elastase (1:500, w/w) and carboxypeptidase (1:100, w/w) in 100 mM PBS (pH 7.0), 37 °C for 1 h.

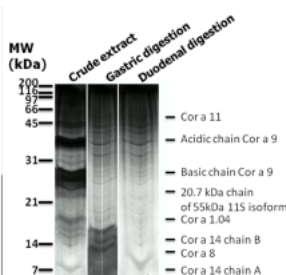
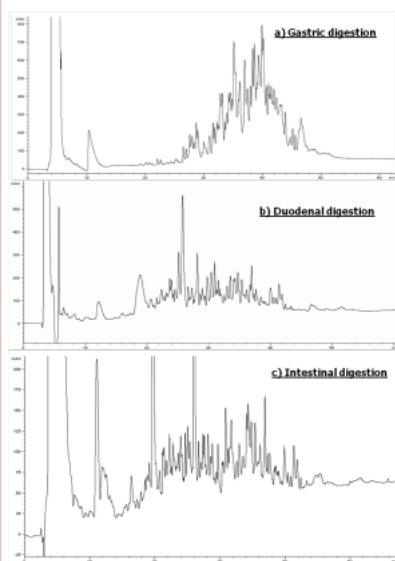
↓  
**INTESTINAL DIGESTION**

Brush Border Membrane BBM enzymes (650 mU/mg) in 100 mM PBS pH 7.2, 37 °C for 1 h.

## Results

### SIMULATED DIGESTION OF CRUDE PROTEINS EXTRACT

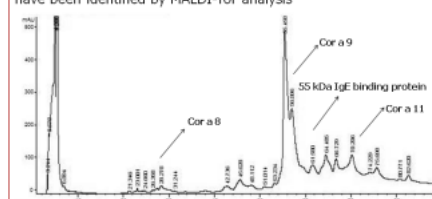
**Fig 1.** Kinetics of sequential release of peptide fragments from crude proteins extract digested by pepsin, pool of pancreatic proteases and BBM monitored by C18 RP HPLC analysis.



**Fig 2.** Comparative SDS digestion profiles of crude protein extract – Proteins were identified by mass fingerprint of peptides derived from in-gel tryptic digestion.

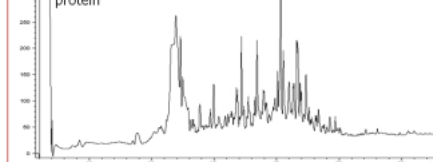
### CRUDE PROTEINS EXTRACT

**Fig 3.** C8 RP-HPLC profile of crude proteins extract – proteins have been identified by MALDI-ToF analysis

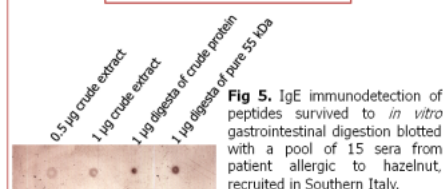


### DIGEST OF 55 KDA 11S GLOBULIN-LIKE ISOFORM

**Fig 4.** C18 RP HPLC profile of peptides delivered by gastrointestinal digestion of pure 55 kDa IgE binding protein



### IMMUNOCHEMICAL ASSAY



**Fig 5.** IgE immunodetection of peptides survived to *in vitro* gastrointestinal digestion blotted with a pool of 15 sera from patient allergic to hazelnut, recruited in Southern Italy.

## Conclusions

Gastrointestinal digestion drastically reduce the most abundant hazelnut allergens: acidic and basic chain of Cor a 9 (11S globulin like protein) and Cor a 11 (48 kDa glycoprotein) Fig 2 . In the gastric phase is evident the increase of low molecular weight fragments (Fig 2 line 2). These was also confirmed by RP-HPLC analysis of peptides (Fig 1).

Dot-blot analysis with sera of allergic patients (Fig 5.) showed that the total peptide digest of crude proteins and pure 55 kDa IgE binding proteins retained IgE-reactivity

Further proteomic investigations are required to identify IgE-binding domains of putative hazelnut allergen(s) resistant to digestion.

Pastorillo EA, Viethe S, Pravettoni V, Farioli L, Trombadori C, Fortunato D, Lutzko D, Colamari M, Anseloni R, Sobilla J, Ballmer-Weber BK, Poulsen UK, Wütrich B, Hansen KS, Robino AM, Ortolani C, Conti A. Identification of hazelnut major allergens in sensitive patients with positive double-blind, placebo-controlled food challenge results. *J Allergy Clin Immunol.* 2002;109(3):563-70.  
Beyer K, Grisham G, Bardine L, Grisham A, Sampson HA. Identification of an 11S globulin as a major hazelnut food allergen in hazelnut induced systemic reactions. *J Allergy and Clin Immunol.* 2002;110(3):517-523.  
Lutzko D et al. Comparison of four variants of a major allergen in hazelnut (*Corylus avellana*) Cor a 1.04 with the major hazelnut pollen allergen Cor a 1.01. *Mol Immunol.* 2002; 38(7):515-25, Jan.

## Knowledgments

I would like to thank my supervisor Pasquale Ferranti, my co-supervisor Gianfranco Mamone and my “colleague” Gianluca Picariello for their constant support, encouragement, their advices and for offering me the opportunity in participating to these interesting projects. I have been extremely lucky in receiving this opportunity to collaborate with them during the last three years.

I have always appreciated all their contributions of ideas and time to make my Ph.D. experience on proteomic-research productive and stimulating. Their delight and enthusiasm on research has always been contagious by strongly motivating me. I learned a lot from their approach about laboratory life, research, how to tackle new problems and how overcome them.

I would like also to thank Raffaele Coppola, director of the ISA-CNR in Avellino for giving me the opportunity to carry out my experimental work to his institute.

I would like to thank prof. Clare Mills for giving me the opportunity to spend a short but very deep period in Norwich to the Institute of Food Research, IFR. During the first phase of my field work she has introduced me to the amazing world of food allergens of plant origin.

I am especially grateful to the INFOGEST cost action for giving me the opportunity to spend two additional months to the IFR. I would like to thank prof. Alan Mackie for his supervision on my work, for his patience and for proving me an exciting and dynamic environment to mature as scientist.

I would like to thank Dr Neil Rigby who, during my two periods spent in Norwich, taught me the passion for research by also answering patiently to any my questions and by helping me in day-by-day laboratory work.

I gratefully acknowledge the funding sources that made my Ph.D. work possible and the constant help and patience of the PhD coordinator Giancarlo Barbieri.

This work has been supported by Italian Ministry of economy and Finance through dedicated grant offered to CNR and ENEA for the project “Innovazione E Sviluppo Del Mezzogiorno - Conoscenze Integrate per la Sostenibilità ed Innovazione del Made in Italy Agroalimentare” Legge n. 191/2009.

*“Life is what happens to you while you're busy making other plans”* and it is what happened me in carrying out my PhD research project.